SUMMARY

We describe the molecular basis of cyclic adenosine monophosphate–stimulated anion secretion in 2-dimensional human duodenal enteroids and show that active chloride and bicarbonate secretion occur in both crypt-like–undifferentiated and villus-like–differentiated enteroids.

BACKGROUND & AIMS: Human enteroids present a novel tool to study human intestinal ion transport physiology and pathophysiology. The present study describes the contributions of Cl− and HCO3− secretion to total cyclic adenosine monophosphate (cAMP)-stimulated electrogenic anion secretion in human duodenal enteroid monolayers and the relevant changes after differentiation.

METHODS: Human duodenal enteroids derived from 4 donors were grown as monolayers and differentiated by a protocol that includes the removal of Wnt3A, R-spondin1, and SB202190 for 5 days. The messenger RNA level and protein expression of selected ion transporters and carbonic anhydrase isoforms were determined by quantitative real-time polymerase chain reaction and immunoblotting, respectively. Undifferentiated and differentiated enteroid monolayers were mounted in the Ussing chamber/voltage-current clamp apparatus, using solutions that contained as well as lacked Cl− and HCO3−/CO2, to determine the magnitude of forskolin-induced short-circuit current change and its sensitivity to specific inhibitors that target selected ion transporters and carbonic anhydrase(s).

RESULTS: Differentiation resulted in a significant reduction in the messenger RNA level and protein expression of cystic fibrosis transmembrane conductance regulator, (CFTR) Na+/K+/2Cl− co-transporter 1 (NKCC1), and potassium channel, voltage gated, subfamily E, regulatory subunit 3 (KCNE3); and, conversely, increase of down-regulated-in-adenoma (DRA), electrogenic Na+/HCO3− co-transporter 1 (NBCe1), carbonic anhydrase 2 (CA2), and carbonic anhydrase 4 (CA4). Both undifferentiated and differentiated enteroids showed active cAMP-stimulated anion secretion that included both Cl− and HCO3− secretion as the magnitude of total active anion secretion was reduced after the removal of extracellular Cl− or HCO3−/CO2. The magnitude of total anion secretion in differentiated enteroids was approximately 33% of that in undifferentiated enteroids, primarily owing to the reduction in Cl− secretion with no significant change in HCO3− secretion. Anion secretion was consistently lower but detectable in differentiated enteroids compared with undifferentiated enteroids in the absence of extracellular Cl− or HCO3−/CO2. Inhibiting CFTR, NKCC1, carbonic anhydrase(s), cAMP-activated K+ channel(s), and Na+/K+−adenosine triphosphatase reduced cAMP-stimulated anion secretion in both undifferentiated and differentiated enteroids.
CONCLUSIONS: Human enteroids recapitulate anion secretion physiology of small intestinal epithelium. Enteroid differentiation is associated with significant alterations in the expression of several ion transporters and carbonic anhydrase isoforms, leading to a reduced but preserved anion secretory phenotype owing to markedly reduced Cl− secretion but no significant change in HCO3− secretion. (Cell Mol Gastroenterol Hepatol 2018;5:591–609; https://doi.org/10.1016/j.jcmgh.2018.02.002)

Keywords: Chloride Secretion; Bicarbonate Secretion; DRA; Ion Transport.

See editorial on page 642.

Ion transport is an essential physiological function of the small intestine. Under physiological conditions, intestinal ion transport is coordinated by intricate regulation of intracellular and extracellular signals.1 Over the past few decades, our knowledge of intestinal ion transport has been revolutionized owing to the application of molecular biology techniques. To date, the critical transporters mediating the absorption and secretion of major ions have been identified and characterized. Nevertheless, further investigation has been limited in a number of aspects, in large part because of the lack of a reliable and complex model system that faithfully recapitulates human intestinal ion transport physiology and pathophysiology. For instance, many observations in animal models are not reproducible in human subjects,2 which is also an important reason for the failure of many drug development efforts that begin with great potential in animal studies but turn out to lack clinical effectiveness and safety.3 In addition, much of our knowledge of intestinal ion transport is based on the findings in nonphysiological cell models such as transfected nonpolarized cells and immortalized cancer cell lines. These results should be interpreted with caution because these models may either show abnormal behavior in particular aspects as a result of genetic instability or fail to present the complex coordination of ion transporters that occurs in the intact human intestine.

To date, the spatial difference in ion transport along the crypt-villus axis of the small intestine remains not fully understood. It is widely believed that absorption and secretion are 2 distinct functions occurring at different sites of the intestinal epithelium, with the former occurring in villi and the latter in crypts. However, a number of studies have suggested that the 2 transport processes may not be completely spatially separated along the crypt-villus axis, and reasons why this separation is unlikely to be so distinct have been described.1,4–7 In addition, a previous study from our group showed the expression and function of Na+/H+ exchanger 3 (NHE3), which is responsible for the majority of electro-neutral sodium absorption in the small intestine and a potential drug target for treating diarrhea/constipation,8 in both crypt-like-undifferentiated enteroids and villus-like-differentiated enteroids, suggesting sodium absorption is not confined to villi.9 Also, several ion transporters that are thought to contribute to anion secretion, including cystic fibrosis transmembrane conductance regulator (CFTR), Na+/K+2Cl− co-transporter 1 (NKCC1), and electrogenic Na+/HCO3− co-transporter 1 (NBCe1), were detectable by immunofluorescence in rat villus enterocytes and co-localized with NHE3.10 CFTR, NBCe1, and, to a lesser extent NKCC1, also were documented as being expressed in human villus enterocytes (Turner JR, unpublished data). Moreover, a chloride-dependent depolarization of apical membrane potential difference was observed upon administration of secretagogues in villi as well as in crypts of rat small intestine.11–13 Hence, it is reasonable to speculate that anion secretion also may occur in villi, although the extent may not be as large as it is in crypts.14 However, this speculation lacks strong evidence from human studies, largely owing to the lack of a reliable method to functionally and physically separate crypt cells and villus cells of human small intestinal epithelium.

Recently, primary cultures of adult stem cell–derived intestinal epithelium, called enteroids, have presented a novel model for studying intestinal health and disease.11–14 Our group has reported the use of 3-dimensional human enteroids as a tool to study ion transport processes and has shown a functional similarity between human enteroids and human small intestine in ion transport physiology and pathophysiology.15 We also confirmed the ability to differentiate enteroids by removing specific ingredients from the culture medium that leads to a separation of undifferentiated enteroids, which have crypt-like properties, and differentiated enteroids, which have villus-like properties, respectively.14,15 This has allowed us to study the spatial difference in cell biology/physiology along the crypt-villus axis of the small intestine. Furthermore, our recent development of 2-dimensional enteroid monolayer cultures has greatly expanded our ability to study enteroids using a variety of approaches, including the Ussing chamber/voltage-current clamp technique for quantitating active electrogenic ion transport processes.13,15,16

In this study, we used the model of human duodenal enteroid monolayers to investigate cyclic adenosine monophosphate (cAMP)-stimulated anion secretion. The aim was to determine the molecular basis of anion secretion and the relevant changes upon enteroid differentiation. We herein report that cAMP-stimulated anion secretion in human duodenal enteroids is composed primarily of Cl− secretion with a smaller component of HCO3− secretion, and is
highly dependent on CFTR, NKCC1, cAMP-activated K⁺ channel(s), Na⁺/K⁺-adenosine triphosphatase (ATPase), and carbonic anhydrase isoforms. Many of these ion transporters and carbonic anhydrase isoforms are subject to regulation by differentiation at both the messenger RNA (mRNA) and protein levels, contributing to a quantitatively reduced but preserved secretory phenotype of differentiated enteroids.

Materials and Methods

This study was approved by the Institutional Review Board of Johns Hopkins University School of Medicine (NA_00038329). All authors had access to the study data and reviewed and approved the final manuscript.

Propagation of Human Enteroid Cultures

The primary cultures of human enteroids were established using a protocol with minor modifications from the method developed by Sato et al.11,17 as previously described. In brief, de-identified specimens of normal human small intestine (duodenum/jejunum/ileum) were obtained during endoscopic or surgical procedures, from which crypts containing adult stem cells were isolated to develop enteroids. Enteroids were embedded in Matrigel (Corning, Tewksbury, MA) in 24-well plates (Corning), and maintained in expansion medium composed of base medium of advanced Dulbecco’s modified Eagle medium/F12 (Life Technologies, Carlsbad, CA) containing 100 U/mL penicillin/streptomycin (Quality Biological, Gaithersburg, MD), 10 mmol/L HEPES (Life Technologies), and 1 × GlutaMAX (Life Technologies), with 50% Wnt3A conditioned medium (produced by L-Wnt3A cell line, ATCC. CRL-2647), 15% R-spondin1-conditioned medium (produced by HEK293T cell line stably expressing mouse R-spondin1; kindly provided by Dr Calvin Kuo, Stanford University, Stanford, CA), 10% Noggin conditioned medium (produced by HEK293T cell line stably expressing mouse Noggin),18,19 1 × B27 supplement (Life Technologies), 1 mmol/L N-acetylcysteine (Sigma-Aldrich, St. Louis, MO), 50 ng/mL human epidermal growth factor (Life Technologies), 1 μg/mL (Leu-15) gastrin (AnaSpec, Fremont, CA), 500 mmol/L A83-01 (Tocris, Bristol, United Kingdom), 10 μmol/L SB202190 (Sigma-Aldrich), and 100 μg/mL primocin (InvivoGen, San Diego, CA). Enteroids were cultured in a 5% CO₂ atmosphere at 37°C and passaged every 7–12 days. Expansion medium was supplemented with 10 μmol/L Y-27632 and CHIR99021 during the first 2 days after seeding. Formation of enteroid monolayers was monitored by morphologic observation using a Zeiss AXIO inverted microscope (Carl Zeiss, Thornwood, NY) and measurement of transepithelial electrical resistance (TER). Once monolayers became confluent, expansion medium was replaced with differentiation medium that was made by substituting Wnt3A, R-spondin1, and SB202190 in the expansion medium with the base medium. Five days later, paired undifferentiated and differentiated enteroid monolayers were studied.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted using the PureLink RNA Mini Kit (Life Technologies) according to the manufacturer’s protocol. Complementary DNA was synthesized from 1 to 2 μg of RNA using SuperScript VILO Master Mix (Life Technologies). Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using Power SYBR Green Master Mix (Life Technologies) on a QuantStudio 12K Flex real-time PCR system (Applied Biosystems, Foster City, CA). Each sample was run in triplicate, and 5 ng RNA-equivalent complementary DNA was used for each reaction. The sequences of gene-specific primers are listed in Table 1. The relative fold changes in mRNA levels of selected ion transporters and carbonic anhydrase isoforms between differentiated enteroids and undifferentiated enteroids (set as 1) were determined using the 2^ΔΔCT method with human 18S ribosomal RNA simultaneously studied and used as the internal control for normalization.

Immunoblotting

Cell pellets were collected from enteroid monolayers and solubilized in lysis buffer containing 60 mmol/L HEPES, 150 mmol/L NaCl, 3 mmol/L KCl, 5 mmol/L EDTA trisodium, 3 mmol/L ethylene glycol-bis(2-aminoethyl)ether)-N, N’, N’-tetraacetic acid (EGTA), 1 mmol/L Na₂PO₄, and 1% Triton X-100 and protease inhibitor cocktail (Sigma-Aldrich), followed by homogenization by sonication. After centrifugation at 5000 g for 10 minutes, the supernatant was collected as the protein lysate. After protein concentration measurement using the bicinchoninic acid method, protein lysates were mixed with 5 × sodium dodecyl sulfate (SDS) buffer and denatured at 70°C for 10 minutes. The protocol was slightly modified for the detection of CFTR and down-regulated-in-adrenona (DRA), in which case protein lysates were mixed with 5 × SDS buffer and incubated at 37°C for 10 minutes. Unless otherwise specified, proteins were separated by SDS–polyacrylamide gel electrophoresis on 4%–20% Mini-Protean TGX Precast Gel (Bio-Rad, Hercules, CA) and transferred onto nitrocellulose membranes (Bio-Rad). After blocking with 5% nonfat milk, blots were probed with primary antibodies overnight at 4°C and IRDye-conjugated secondary antibodies against rabbit and mouse immunoglobulin G (LI-COR, Lincoln, NE) for 1 hour at room temperature. Finally, blots were scanned using an Odyssey CLx imaging system (LI-COR) and the protein bands were visualized and quantified using Image Studio software (LI-COR). The primary antibodies used in this study are listed in Table 2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the loading control.
Table 1. Gene-Specific Primers for qRT-PCR

| Gene  | Forward (5’-3’)                      | Reverse (5’-3’)                      |
|-------|-------------------------------------|-------------------------------------|
| AE2   | TCCCTCTCTTCCGCGAGT                  | TGCTGGTCACGATCCAGAA                 |
| ATP1A1| ACAGACTTGGAGCGGGAGGTATT            | TCCATATTGAGATGTGGGAG                |
| ATP1B1| CCGTTGCGATGGTGGTTAAAGA             | GCATCATTGGATGCTGGCAGA               |
| CA1   | TTGAGAGAAAGGATCCGATCA              | CTACGTGAAGTCGAGCAAG                  |
| CA2   | CACCCTCTCCTCTTGAGAT               | AGTTTACGGAATTTAACAGGTTCTGGT         |
| CA4   | CTGGTGCTAGGAGTGGTCAAGC            | GAAGAAGAAAGGCTCCAGATTT              |
| CA9   | CTTTTCAGAAGGTGAGGAGG              | GCAAAGTCAGGTGGAGAAGA                 |
| CFTR  | GAAGAAGGAGTGGAGAAGTGTGGTGAAGC     | GCTTTCTCAGAATATTTCCAGAAAA           |
| DRA   | CCATCATGTCGATGTGATTGC             | AGCTCGAGGACGAGCTTT                  |
| GATA4 | GAAGGAGCAGCAGAAGACTGAG             | GGTGCTGAGGATTTCCAGGAA               |
| KCNE3 | TTAAGGAGTGGTCTGGTACTG             | ATGCAACAGGATCGGTGCTTT               |
| KCNQ1 | GCTTTCTCTTATAAAACGTGGAGA          | GAAGACAGGATGGAGACAGTT               |
| KI67  | GAGGTGGTCAGAAAATCCAAAA            | GTCTGGATGCTGCTGTTGT                 |
| LGR5  | ACCAGACTGACCTTTCGGAAC             | TTTCCAGAGGATGGTGCTT                 |
| NBCe1 | CCTCAAGCATTGTTGATGTA              | AACTCTGGGCGACAGACGTG                |
| NBCn1 | GCAAGAACACCATTCGCTCCCTCA          | GCTTCCACACCTCATTCCATGCTC            |
| NHE1  | CTTTCACCACGTCTTGTGGAG             | ATGAGAGGCGTGTCTGCTT                 |
| NHE2  | CTCTCCACTCTGACGGCATG              | GCTGTATGCCCATCAGA                   |
| NHE3  | GTCTTCCTGACGGTCTTCA               | ATGAGAATAGCAGGCAAGC                 |
| NKCC1 | AAAGAGAAGTCTGCAGACG               | CTAGAAGAAGCAGCCTTGGT                |
| PAT-1 | TCTACAGGTAGTCTGTCAGAGGA           | GAGAGGATAGGCTGCTCAGAAGG             |
| S1    | TTTGGCGATCCCAAGTTGCA              | ATCCAGGCGACAGCAAGAC                 |
| 18S   | GCAATTATCCCCATGAGG                | GGAACCTTAATCAACGCGAAGC              |

ATP1A1, Na\(^+\)/K\(^+\)–adenosine triphosphatase \(\alpha\)-1 subunit; ATP1B1, Na\(^+\)/K\(^+\)–adenosine triphosphatase \(\beta\)-1 subunit; KCNE3, potassium channel, voltage gated, subfamily E, regulatory subunit 3; KCNQ1, potassium channel, voltage gated, subfamily Q, member 1; LGR5, leucine-rich repeat-containing G-protein–coupled receptor 5; NBCn1, electroneutral Na\(^+\)/HCO\(_3\)\(^-\) co-transporter 1; PAT-1, putative anion transporter 1.

Transepithelial Electrical Resistance
TER was measured using an EVOM2 epithelial voltohmmeter (World Precision Instruments, Sarasota, FL) before refreshing the medium. The readings of the voltohmmeter were normalized by the surface area of Transwell inserts (0.33 cm\(^2\)) to calculate the unit area of resistance (Ω cm\(^2\)).

Ussing Chamber/Short-Circuit Current Measurement
Transwell inserts carrying enteroid monolayers were mounted in Ussing chambers (Physiological Instruments, San Diego, CA). The apical and basolateral hemichambers were filled with buffer that was gassed continuously with 95% O\(_2\)/5% CO\(_2\), maintained at 37°C, and connected

Table 2. Primary Antibodies for Immunoblotting

| Antigen | Manufacturer/Source | Catalog number | Host | Dilution |
|---------|---------------------|---------------|------|----------|
| ATP1A1  | Developmental Studies Hybridoma Bank (Iowa City, IA) | a5           | Mouse | 1:1000   |
| CA2     | Novus (Littleton, CO) | NB600-919     | Rabbit | 1:500    |
| CFTR    | Cystic Fibrosis Foundation Therapeutics (Chapel Hill, NC) | 217     | Mouse | 1:400    |
| DRA     | Santa Cruz          | sc-376187     | Mouse | 1:200    |
| GAPDH   | Sigma-Aldrich       | G8795         | Mouse | 1:5000   |
| NBCe1   | Abcam (Cambridge, MA) | ab30322      | Rabbit | 1:500    |
| NHE2    | Provided by Dr Chung-Ming Tse (Johns Hopkins University, Baltimore, MD) | N/A | Rabbit | 1:500    |
| NHE3    | Novus (Littleton, CO) | NBPA-85274 | Rabbit | 1:1000   |
| NKCC1   | Developmental Studies Hybridoma Bank (Iowa City, IA) | T4-C | Mouse | 1:1000   |

ATP1A1, Na\(^+\)/K\(^+\)–adenosine triphosphatase \(\alpha\)-1 subunit; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; N/A, not applicable.
to a voltage-current clamp apparatus (Physiological Instruments) via Ag/AgCl electrodes and 3 mol/L KCl agar bridges. Krebs–Ringer bicarbonate buffer (KRB buffer) consisted of 115 mmol/L NaCl, 25 mmol/L NaHCO3, 0.4 mmol/L KH2PO4, 2.4 mmol/L K2HPO4, 1.2 mmol/L CaCl2, 1.2 mmol/L MgCl2, pH 7.4. In Cl−-free buffer and HCO3−-free buffer, Cl− and HCO3− in KRB buffer were replaced with glucocon. Cl−-free buffer was supplemented with an additional 5 mmol/L calcium gluconate to maintain the level of free calcium. HCO3−-free buffer was gassed with 100% O2 and supplemented with 5 mmol/L HEPEs and 1 mmol/L acetazolamide to inhibit the endogenous production of HCO3−/CO2. In addition, buffer in the basolateral hemichamber was supplemented with 10 mmol/L glucose as an energy substrate; buffer in the apical hemichamber was supplemented with 10 mmol/L mannitol to maintain the osmotic balance. Current clamping was used, and short-circuit current (Isc) and TER were recorded every 20 seconds by Acquire and Analyze software (Physiological Instruments). To investigate the change of short-circuit current (ΔIsc) in response to an apical-basolateral gradient of glucose, 40 mmol/L glucose was added to the apical hemichamber with 40 mmol/L mannitol to the basolateral hemichamber. To investigate cAMP-stimulated anion secretion, 10 µmol/L forskolin was added to the basolateral hemichamber, after which the effects of inhibitors targeting selected ion transporters and carbonic anhydrase(s) were studied (Table 3). The time-course changes in Isc and TER were delineated.

### Results

#### Phenotypic Changes After Differentiation

In previous studies, we reported a 5-day differentiation protocol of human enteroids that allowed the transition of undifferentiated enteroids consisting of stem cells, Paneth cells, and transit-amplifying cells into differentiated enteroids made up primarily of enterocytes, goblet cells, and enteroendocrine cells. In the present study, we further characterized the phenotypic changes in 2-dimensional human duodenal enteroids after 5 days of differentiation. First, there was an increase in TER upon differentiation (Figure 1A and B); 5-day differentiated enteroid monolayers had significantly higher TER than undifferentiated enteroid monolayers (1167 ± 140 Ωcm2 vs 371 ± 29 Ωcm2; P < .001), but prolonged differentiation up to 7 days did not cause an additional increase in TER. This is in accordance with our previous observation in human jejunal enteroid monolayers. Second, differentiated enteroid monolayers showed a higher response rate to an apical-basolateral gradient of glucose than undifferentiated enteroid monolayers in the magnitude of maximal increase in Isc (15.6 ± 2.9 µA/cm2 vs 7.8 ± 1.5 µA/cm2; P < .001) (Figure 1C and D), indicating an increase in the apical Na+–glucose co-transport activity after differentiation. Third, Sucrase-isomaltase (SI), a brush-border glucosidase enzyme and intestinal differentiation marker, was up-regulated significantly at the mRNA level upon differentiation (Figure 1E). Fourth, the mRNA levels of leucine-rich repeat-containing G-protein–coupled receptor 5 (LGR5), an intestinal stem cell marker, and Ki67, a cellular proliferation marker, were dramatically reduced with differentiation (Figure 1F and G).

In addition, studies were performed to characterize the segment-specific identity of duodenal enteroid monolayers before and after differentiation. GATA4 is an intestinal segment-specific transcription factor that controls the cephalocaudal expression pattern of small intestine by repressing the expression of ileum-specific genes in upper small intestinal segments. The current study found that GATA4 mRNA was confined to human enteroid monolayers derived from duodenum and jejunum, and its amount was maintained in duodenum and slightly increased in jejunum after differentiation (Figure 1H). Taken together, these results present additional evidence that the 5-day differentiation protocol allows the separation of crypt-like cells (undifferentiated enteroids) and villus-like cells (differentiated enteroids) without affecting their segment-specific identity in 2-dimensional human duodenal enteroids, encouraging the subsequent investigations on the differentiation-associated modulations of ion transport.

#### Expression of Ion Transporters and Carbonic Anhydrase Isoforms Upon Differentiation

To investigate the alterations of ion transport after differentiation, mRNA levels of selected ion transporters and carbonic anhydrase isoforms were examined in several cases, experimental variability within single enteroid lines was calculated as means ± SEM.

### Table 3. Compounds for Using Chamber/Short-Circuit Current Measurement

| Compound          | Manufacturer     | Final concentration Side |
|-------------------|------------------|--------------------------|
| Acetazolamide     | Sigma-Aldrich    | 250 µmol/L AP + BL       |
| Bumetanide        | Sigma-Aldrich    | 100 µmol/L BL            |
| CFTRinh-172       | EMD Millipore    | 5 or 25 µmol/L AP        |
| Chromanol 293B    | Sigma-Aldrich    | 10 µmol/L BL             |
| Forskolin         | Sigma-Aldrich    | 10 µmol/L BL             |
| Ouabain           | Sigma-Aldrich    | 100 µmol/L BL            |
| SO859             | Sigma-Aldrich    | 30 µmol/L BL             |
| SITS              | Sigma-Aldrich    | 1 mmol/L BL              |
| Tenapanor         | Ardelyx (Fremont, CA) | 0.1–1 µmol/L AP   |

AP, apical; BL, basolateral.
Figure 1. Phenotypic changes of human enteroid monolayers upon differentiation. (A) TER of undifferentiated (UD) and differentiated (DF) duodenal enteroid monolayers over time. Differentiated enteroids had a significantly higher TER than undifferentiated enteroids on day 4 (*P < .05) and day 5 (**P < .001). n = 6 experiments with paired enteroid monolayers derived from 3 donors. (B) There was no difference in TER between duodenal enteroid monolayers that were differentiated for 5 days or 7 days (1162 ± 246 Ω·cm² vs 1042 ± 193 Ω·cm², P = .66). **P < .05. n = 3 experiments with paired enteroid monolayers derived from 3 donors. (C and D) A representative trace and quantitative analysis showing a higher response in ΔUᵣ to an apical-basolateral gradient of glucose (30 mmol/L) in differentiated enteroids than undifferentiated enteroids (***P < .001). n = 4 experiments with paired enteroid monolayers derived from 4 donors. (E–G) qRT-PCR data showing the mRNA level of sucrase-isomaltase (SI) was increased by 15.4 ± 4.6 times upon differentiation, whereas that of leucine-rich, repeat-containing, G-protein–coupled receptor 5 (LGR5) and K67 was reduced by 379 ± 209 and 102 ± 27 times, respectively. **P < .01, ***P < .001. n = 4–8 experiments with paired enteroid monolayers derived from 4 donors. (H) qRT-PCR data showing that GATA4 gene expression was confined to duodenal and jejunal enteroid monolayers with no change in duodenum and a slight increase in jejenum after differentiation. **P < .01. n.d., not detected (threshold [Ct] value >34). n = 3 experiments with paired enteroid monolayers derived from 3 donors (duodenum, jejunum) or 2 donors (ileum).

We further studied the protein expression of selected ion transporters and carbonic anhydrase isoforms in undifferentiated and differentiated duodenal enteroid monolayers by immunoblotting (Figure 4A and B). Consistent with the results of qRT-PCR, CFTR (2.0-fold) and NKCC1 (4.0-fold) were down-regulated significantly at the protein level, CA2 (2.5-fold), DRA (4.6-fold) and NBCe1 (2.3-fold) were up-regulated significantly, and ATP1A1 and NHE2 were not changed upon differentiation. Although there was no significant change at the mRNA level, the protein expression of NHE3 was slightly but significantly up-regulated by 1.5-fold after differentiation. In sum, these data suggest a significant regulatory effect of differentiation on the expression of multiple ion transporters and carbonic anhydrase isoforms, some of which are known to be essential for intestinal Cl⁻ and HCO₃⁻ secretion.

Reduced cAMP-Stimulated Anion Secretion Upon Differentiation

To study anion secretion at the functional level, Transwell inserts carrying paired undifferentiated and differentiated enteroid monolayers were mounted in Ussing chambers, and forskolin was used to induce cAMP-stimulated electrogenic anion secretion as indicated by the increase in short-circuit current. As shown in Figure 5A and D,

undifferentiated and differentiated duodenal enteroid monolayers by qRT-PCR. The ion transporters and carbonic anhydrase isoforms selected are known to play important roles in Cl⁻ and HCO₃⁻ secretion, electroneutral Na⁺ absorption, and intracellular pH regulation under physiological and pathophysiological conditions. As shown in Figure 2A, several ion transporters and carbonic anhydrase isoforms were up-regulated significantly at the mRNA level upon differentiation. These included carbonic anhydrase (CA)4 (64.4-fold), DRA (21.6-fold), CA2 (4.0-fold), NHE1 (2.7-fold), NBCe1 (2.4-fold), and Na⁺/K⁺-adenosine triphosphatase β-1 subunit (ATP1B1) (1.7-fold). In contrast, several ion transporters were down-regulated significantly after differentiation, including NKCC1 (7.1-fold), potassium channel, voltage gated, subfamily E, regulatory subunit 3 (KCN3) (5.2-fold), and CFTR (2.7-fold). The mRNA levels of the following ion transporters and carbonic anhydrase isoforms were not significantly changed by differentiation: anion exchanger 2 (AE2), Na⁺/K⁺-adenosine triphosphatase α-1 subunit (ATP1A1), CA1, CA9, potassium channel, voltage gated, subfamily Q, member 1 (KCNQ1), electroneutral Na⁺/HCO₃⁻ co-transporter 1 (NBCe1), NHE2, NHE3, and putative anion transporter 1 (PAT-1). The qRT-PCR threshold values are shown in Figure 2B. Most of these findings in 2-dimensional enteroids were consistent with the results obtained from in parallel studies of 3-dimensional enteroids (Figure 3).
in KRB buffer in which extracellular Cl- and HCO$_3$/CO$_2$ are both present, forskolin caused an immediate increase in I$_{sc}$ in both undifferentiated enteroids (34.8 ± 4.6 μA/cm$^2$) and differentiated enteroids (10.4 ± 1.3 μA/cm$^2$). Based on the existing knowledge of cAMP/protein kinase A–induced anion secretion, we speculated that cAMP-stimulated anion secretion in KRB buffer consisted of 2 major components, namely Cl- secretion and HCO$_3$ secretion, and that each component was largely dependent on the presence of extracellular Cl- and HCO$_3$/CO$_2$, respectively. As such, we investigated whether the removal of extracellular Cl- or HCO$_3$/CO$_2$ reduced cAMP-stimulated secretion. Figure 5B and C show that duodenal enteroid monolayers had a smaller response to forskolin in HCO$_3$-free buffer and Cl- free buffer. Quantitatively, the magnitude of forskolin-induced ΔI$_{sc}$ was 27.7 ± 4.8 μA/cm$^2$ in HCO$_3$-free buffer and 3.3 ± 0.6 μA/cm$^2$ in Cl- free buffer for undifferentiated enteroids, and was 3.9 ± 0.9 μA/cm$^2$ in HCO$_3$-free buffer and 0.5 ± 0.1 μA/cm$^2$ in Cl- free buffer for differentiated enteroids (Figure 5D). Figure 5E shows the experimental variability by showing the means ± SEM of the maximal forskolin response of 4 separate enteroid lines in KRB, HCO$_3$-free, and Cl- free buffer. We further normalized the magnitude of forskolin-induced ΔI$_{sc}$ in HCO$_3$-free and Cl- free buffer to that in KRB buffer (set as 100%). As shown in Figure 5F, there was a reduction to 80% ± 7% in HCO$_3$-free buffer and 9% ± 2% in Cl- free buffer for undifferentiated enteroids, and 41% ± 8% in HCO$_3$-free buffer and 5% ± 2% in Cl- free buffer for differentiated enteroids (these data were calculated by considering all experiments within a single enteroid line as n = 1 and are shown as the means ± SEM of 4 enteroid lines). These results support the speculation that when extracellular Cl- and HCO$_3$/CO$_2$ are both available, the cAMP-stimulated anion secretion from enteroid monolayers comprises both Cl- and HCO$_3$ secretion. Furthermore, these results indicate a higher dependency of cAMP-stimulated anion secretion on extracellular Cl- than on extracellular HCO$_3$/CO$_2$.

We also compared undifferentiated and differentiated enteroids in their response to forskolin in multiple buffers. In KRB buffer, the maximal increase of I$_{sc}$ in response to forskolin was significantly lower in differentiated enteroids, which was 33% ± 4% of that of undifferentiated enteroids (P < .001) (Figure 5G). This is in contrast to our previous finding in 3-dimensional duodenal enteroids that forskolin-induced swelling was greater in differentiated than undifferentiated enteroids.9 Notably, we have changed the nature of expansion medium for enteroids since the initial report9 and used the modified expansion medium which lacks nicotinamide in the current study (see the Materials and Methods section).22 As shown in Figure 5J, when studies were repeated using the modified expansion medium, forskolin-induced swelling was greater in undifferentiated than differentiated 3-dimensional duodenal enteroids. In the present study, the magnitude of forskolin-induced ΔI$_{sc}$ in duodenal enteroid monolayers was consistently lower in differentiated enteroids compared with undifferentiated enteroids in multiple buffer conditions: 16% ± 3% of that of undifferentiated enteroids in HCO$_3$-free buffer (P < .001) (Figure 5H) and 15% ± 2% in Cl- free buffer (P < .001) (Figure 5I). These results show reduced anion secretion after differentiation; however, forskolin-induced ΔI$_{sc}$ was still detectable in differentiated enteroids in all buffer conditions, suggesting a preserved anion secretory phenotype in differentiated enteroids.

**Apical Ion Transporters in cAMP-Stimulated Anion Secretion**

Anion secretion involves multiple ion transporters that are located on the apical and basolateral membranes of intestinal epithelial cells. The contribution of selected ion transporters to cAMP-stimulated anion secretion was determined using specific inhibitors, and the effects of these inhibitors on TER were studied simultaneously (Figure 6). CFTR is an apical ion channel that is permeable to both Cl- and, to a lesser extent, HCO$_3$.23 A previous study showed that blocking CFTR resulted in substantial inhibition of forskolin-induced ΔI$_{sc}$ as well as luminal fluid accumulation in jejunal enteroids.24 In this study, we further confirmed the role of CFTR in cAMP-stimulated anion secretion of duodenal enteroid monolayers using CFTRinh-172 as a CFTR blocker. In KRB buffer, CFTRinh-172 (25 μmol/L) inhibited 85% ± 3% and 95% ± 8% of forskolin-induced ΔI$_{sc}$ in undifferentiated and differentiated enteroids, respectively (Figure 7A and D). A lower dose of CFTRinh-172 (5 μmol/L) also inhibited the majority of forskolin-induced ΔI$_{sc}$ in KRB buffer (84% ± 6% for undifferentiated enteroids and 87% ± 6% for differentiated enteroids) (Figure 7E). Similarly, the inhibitory effect of CFTRinh-172 (25 μmol/L) of 90%-119% of the forskolin-induced ΔI$_{sc}$ was observed in HCO$_3$-free buffer and Cl- free buffer (Figure 7B–D).

A previous study by our group reported a potential role of NHE3 in forskolin-induced swelling in 3-dimensional duodenal enteroids, but it was not clear whether NHE3 participates in this process through its role in Na$^+$ absorption, anion secretion, or both.9 To address this, we studied the effect of tenapanor, a novel NHE3 inhibitor with a median inhibitory concentration in the nanomolar range,1 on forskolin-induced ΔI$_{sc}$ in duodenal enteroid monolayers. As shown in Figure 7F, the addition of up to 1 μmol/L tenapanor did not have any significant effect on forskolin-induced ΔI$_{sc}$ in either undifferentiated or differentiated enteroids in KRB buffer. Therefore, NHE3 does not appear to contribute to cAMP-stimulated anion secretion in duodenal enteroids.

**Basolateral Ion Transporters and Carbonic Anhydrase(s) in cAMP-Stimulated Anion Secretion**

A number of compounds that target selected basolateral ion transporters and carbonic anhydrase(s) were used to determine their roles in cAMP-stimulated anion secretion. In KRB buffer, administration of bumetanide, a specific blocker of NKCC1, caused a dramatic reduction in forskolin-induced ΔI$_{sc}$ in undifferentiated enteroids and a lesser reduction in...
Figure 3. mRNA level of selected ion transporters and carbonic anhydrase isoforms in 3-dimensional human duodenal enteroids. The mRNA levels of selected ion transporters and carbonic anhydrase isoforms were determined by qRT-PCR and the relative fold changes between differentiated (DF) and undifferentiated (UD) 3-dimensional enteroids (set as 1) were calculated using 18S ribosomal RNA as the endogenous control. These data are mostly consistent with those of parallel studies in duodenal enteroid monolayers (See results in Figure 2). *P < .05, **P < .01, ***P < .001. n = 5 experiments with paired 3-dimensional enteroids derived from 5 donors.

Figure 2. (See previous page). mRNA levels of selected ion transporters and carbonic anhydrase isoforms in human duodenal enteroid monolayers. (A) The mRNA levels of selected ion transporters and carbonic anhydrase isoforms were determined by qRT-PCR and the relative fold changes between differentiated (DF) and undifferentiated (UD) enteroid monolayers (set as 1) were calculated using 18S ribosomal RNA as the endogenous control. Most of the results are consistent with those in parallel studies of 3-dimensional duodenal enteroids (See results in Figure 3). *P < .05, **P < .01, ***P < .001. n = 4–8 experiments with paired enteroid monolayers derived from 4 donors. (B) Scatter plots showing the threshold [Ct] values of selected genes in undifferentiated and differentiated duodenal enteroid monolayers. Each plot represents the result of a single experiment. A total of 5 ng RNA-equivalent complementary DNA was used for each qRT-PCR reaction. These Ct values were further normalized to 18S ribosomal RNA (Ct value ~7–8) to determine the relative amount of each gene in each sample. n = 4–8 experiments with paired enteroid monolayers derived from 4 donors.
differentiated enteroids (Figure 8A). In most cases, a bumetanide-insensitive component was observed, particularly in differentiated enteroids. We then studied whether this bumetanide-insensitive component could be inhibited by acetazolamide, a general carbonic anhydrase inhibitor. As shown in Figure 8A and B, the bumetanide-insensitive component was sensitive to acetazolamide, and the combination of bumetanide and acetazolamide abolished forskolin-induced \( \Delta I_{\text{sc}} \) regardless of their order of addition. These results confirm that forskolin-induced \( \Delta I_{\text{sc}} \) in KRB buffer is contributed by Cl\(^-\) secretion and HCO\(_3\)\(^-\) secretion as 2 major components, and suggest the involvement of NKCC1 and carbonic anhydrase(s) in cAMP-stimulated anion secretion through their roles in Cl\(^-\) secretion and HCO\(_3\)\(^-\) secretion, respectively.

We further compared undifferentiated and differentiated enteroids in their sensitivity to bumetanide and acetazolamide (Figure 8C and D). The bumetanide-sensitive component accounted for a relatively greater percentage in undifferentiated enteroids than differentiated enteroids.
enteroids (75% ± 3% vs 44% ± 5%; P < 0.001), whereas the acetazolamide-sensitive component contributed to a relatively larger percentage in differentiated enteroids than undifferentiated enteroids (51% ± 8% vs 13% ± 2%; P < 0.001). We also calculated the absolute reduction in forskolin-induced $\Delta I_{sc}$ after the addition of acetazolamide (Figure 8F). This represents the acetazolamide-sensitive anion secretion and was very similar between undifferentiated enteroids and differentiated enteroids (4.4 ± 0.8 $\mu$A/cm² vs 5.3 ± 0.9 $\mu$A/cm², P = 0.35).

In addition, we found that cAMP-stimulated anion secretion in duodenal enteroid monolayers required active Na$^+$/K$^+$/ATPase because ouabain caused a decrease in forskolin-induced $\Delta I_{sc}$ with a rapid onset within 2 minutes of addition (Figure 8F). Moreover, although several previous publications have suggested a potential role of basolateral anion exchangers such as AE2 and sodium/bicarbonate co-transporters such as NBCe1 in anion secretion, the present study failed to validate the functional involvement of these transporters in cAMP-stimulated anion secretion in human duodenal enteroids because there was no evident inhibition of forskolin-induced $\Delta I_{sc}$ after the administration of disodium 4-acetamido-4'-isothiocyanato-stilben-2,2'-disulfonate (SITS), which inhibits AE2, or S0859, which inhibits the NBC family (Figure 8G and H).

### Basolateral Ion Transporters in cAMP-Stimulated $Cl^-$ Secretion

We also studied the functional involvement of several basolateral ion transporters in HCO$_3^-$-free buffer, in which forskolin-induced $\Delta I_{sc}$ was completely attributed to electrogenic $Cl^-$ secretion via CFTR. In the absence of HCO$_3^-$, inhibition of NKCC1 by bumetanide abolished forskolin-induced $\Delta I_{sc}$ in both undifferentiated enteroids (97% ± 3%) and differentiated enteroids (120% ± 10%), verifying the essential role of NKCC1 as a basolateral $Cl^-$ loader (Figure 9A and B). In addition, the extensive and rapid-onset inhibitory effect of ouabain also occurred in HCO$_3^-$-free buffer (Figure 9C). Finally, we found that 57% ± 13% and 38% ± 8% of the forskolin-induced $\Delta I_{sc}$ in undifferentiated and differentiated enteroids, respectively, was inhibited by chromanol 293B, indicating the dependence of $Cl^-$ secretion on basolateral cAMP-activated $K^+$ channel(s) (Figure 9D and E).

### Discussion

The present study documents the changes in cAMP-stimulated anion secretion and expression of relevant ion transporters during differentiation of normal human duodenal enteroid monolayers, showing the following: (1) similarities with the previous characterization of 3-dimensional human duodenal enteroids, although the changes in expansion medium seem to cause changes in some aspects of the secretory processes; (2) that cAMP-stimulated anion secretion occurs in both undifferentiated and differentiated enteroid monolayers, further supporting that ion transport processes in human undifferentiated and differentiated enteroids are more quantitatively than qualitatively different; (3) that both undifferentiated and differentiated enteroids perform what appears to be cAMP-stimulated $Cl^-$ secretion and HCO$_3^-$ secretion, with $Cl^-$ secretion being predominant; and (4) that although total anion secretion is much less in differentiated enteroids, the extent of HCO$_3^-$ secretion is similar in differentiated and undifferentiated enteroids.

Initially, we showed that the removal of Wnt3A/R-spondin1/SB202190 induced differentiation in duodenal enteroid monolayers, as supported by several phenotypic changes characterized at day 5. Comparison between undifferentiated and differentiated enteroid monolayers also showed several differentiation-related alterations in the expression of multiple ion transporters and carbonic anhydrase isoforms, consistent with the results based on studies of intact human (Turner JR, unpublished data) and animal small intestine. These data support our ability to separately study intestinal epithelial cells that represent crypts and villi using undifferentiated and differentiated enteroids. However, where along the villus our differentiation protocol is modeling is difficult to state given that not all villus cells are differentiated to the same extent, presumably
continuing to differentiate as they move from the base of villus to the tip. Moreover, data are lacking that show the differentiation state and expression of transport proteins in epithelial cells at multiple positions along the human intestinal villus. Similarly, although the crypt base has LGR5-positive stem cells, Paneth cells, and transit-amplifying cells that proliferate, it is likely that cells of multiple states of differentiation are present, particularly at the upper crypt. Importantly, it is not yet known where in the crypt the anion secretory cells appear.

A major contribution of this study is the identification of the transport processes and cell populations that contribute to small intestinal anion secretion stimulated by increased intracellular cAMP. Although the functional relevance of enteroids to understanding human intestinal ion transport physiology has been shown by several previous studies,9,24 the characterization of human intestinal anion secretory
processes remains incomplete. In the present study, we defined active electrogenic anion secretion stimulated by forskolin in human duodenal enteroid monolayers by the Ussing chamber/voltage-current clamp technique. Specifically, this includes cAMP-stimulated Cl\(^-\) secretion (HCO\(_3\)\(^-\)-independent and HCO\(_3\)\(^-\)-dependent) and HCO\(_3\)\(^-\) secretion (Cl\(^-\)-independent and Cl\(^-\)-dependent). It is important to point out that only electrogenic ion transport is defined by the approach of measuring \(I_{sc}\); this did not allow us to specifically quantify the electroneutral Cl\(^-\)-dependent HCO\(_3\)\(^-\) secretion that is provided by an apical anion exchanger, such as DRA, which exchanges Cl\(^-\) that is secreted by CFTR for intracellular HCO\(_3\)\(^-\), and that together with the linked CFTR-related Cl\(^-\) secretion is an overall electrogenic process.\(^{28}\) Similarly, the specific contribution of HCO\(_3\)\(^-\)-dependent Cl\(^-\) secretion, which includes the putative activated DRA stimulation of CFTR activity, could not be determined.\(^{29,30}\) nor is it known if it occurs in intact mammalian small intestine. In addition, the current study was not able to simultaneously quantitate the amount of active HCO\(_3\)\(^-\) transport, given that our available equipment could not accomplish simultaneous pH titration and short-circuiting. In addition, although we have found many similarities in cAMP-stimulated secretion between enteroids derived from duodenum and jejunum, the findings of the current study in duodenal enteroids may not be completely extrapolated to other small intestinal segments given the special functions of each segment.

cAMP-stimulated anion secretion was present in both the crypt-like–undifferentiated enteroids and the villus-like–differentiated enteroids with the following characteristics. First, the magnitude of forskolin-stimulated anion secretion in differentiated enteroids was approximately 33% of that in undifferentiated enteroids. Second, a reduction in the magnitude of forskolin-induced \(\Delta I_{sc}\) was observed in both undifferentiated and differentiated enteroids after the removal of extracellular Cl\(^-\), presumably owing to loss of Cl\(^-\) secretion and also Cl\(^-\)-dependent HCO\(_3\)\(^-\) secretion. Similarly, the removal of extracellular HCO\(_3\)/CO\(_2\), which causes loss of HCO\(_3\)\(^-\) secretion and HCO\(_3\)-dependent Cl\(^-\) secretion, resulted in reduced magnitude of forskolin-induced \(\Delta I_{sc}\), but this was modest compared with that with extracellular Cl\(^-\) removal. Similar effects of extracellular anion removal also were found in mouse duodenum.\(^{21,31}\) Although the cAMP-stimulated anion secretion in the absence of extracellular Cl\(^-\) or HCO\(_3\)/CO\(_2\) does not represent the entire HCO\(_3\)\(^-\) or Cl\(^-\) secretion, this method of extracellular anion removal at least allowed us to confirm the dependency of cAMP-stimulated anion secretion on extracellular anions. Third, Cl\(^-\) secretion, primarily defined as the bumetanide-sensitive component of forskolin-induced \(\Delta I_{sc}\), makes up the bulk of anion secretion in undifferentiated enteroids and a significant part in differentiated enteroids. Specifically, 75% of the forskolin-induced \(\Delta I_{sc}\) was inhibited by bumetanide in undifferentiated enteroids and 44% in differentiated enteroids. This is consistent with the in vivo observation that bumetanide inhibited a large part of forskolin-induced \(\Delta I_{sc}\) without affecting bicarbonate secretion in mouse duodenum.\(^{32}\) Fourth, a bumetanide-insensitive component of forskolin-induced \(\Delta I_{sc}\) was observed in both undifferentiated and differentiated enteroids. This residual component is either Cl\(^-\) secretion dependent on another bumetanide-insensitive basolateral Cl\(^-\) uptake pathway or HCO\(_3\)\(^-\) secretion. The former has not been identified in the small intestine. Also, although the basolateral anion exchanger AE2, which is present similarly in undifferentiated and differentiated enteroids, could provide such a transport pathway,\(^{25,33}\) the lack of effect on forskolin-induced \(\Delta I_{sc}\) by blocking AE2 with STS suggests it unlikely to be involved. Of note, this bumetanide-insensitive

Figure 5. (See previous page). cAMP-stimulated electrogenic anion secretion in human duodenal enteroid monolayers. (A–C) Representative traces showing forskolin-induced \(\Delta I_{sc}\) arising from undifferentiated (UD) and differentiated (DF) duodenal enteroid monolayers in (A) KRB buffer, (B) HCO\(_3\)\(^-\)-free buffer, and (C) Cl\(^-\)-free buffer. In all experiments, forskolin (10 \(\mu\)mol/L) was added to the basolateral side to induce anion secretion. (D) Scatter plots showing the magnitude of forskolin-induced \(\Delta I_{sc}\) for undifferentiated enteroids and differentiated enteroids in multiple buffer conditions. Each plot represents the result of a single experiment (\(n = 1–3\) enteroid monolayers). Statistical analysis was performed using an unpaired \(t\) test. **\(P < .001\), \(n = 11–19\) experiments with paired enteroid monolayers derived from 4 donors. (E) Scatter plots showing the individual data of each enteroid line from Figure 4D. Each plot represents the result of a single experiment (\(n = 1–3\) enteroid monolayers). Each color represents a single enteroid line derived from an individual donor. Note the moderate variability in the magnitude of forskolin-induced \(\Delta I_{sc}\) between enteroid lines derived from different donors and between experiments within each single enteroid line. The **horizontal lines** are the means, and the standard error bars show the experimental variability within each single enteroid line. \(n = 11–19\) experiments with paired enteroid monolayers derived from 4 donors. (F) Quantitative analysis comparing the magnitude of forskolin-induced \(\Delta I_{sc}\) in HCO\(_3\)\(^-\)-free buffer and Cl\(^-\)-free buffer with that in KRB buffer (set as 100%). In both undifferentiated and differentiated enteroids, there was a significant reduction in forskolin-induced \(\Delta I_{sc}\) after the removal of extracellular HCO\(_3\)/CO\(_2\) and an even more pronounced reduction after the removal of extracellular Cl\(^-\). \(P < .05\), **\(< .01\), ***\(< .001\). \(n = 4\) enteroid lines derived from 4 separate donors (the results were first analyzed within each single enteroid line and the averages of each enteroid line subsequently were combined for statistical analysis considering the total number of enteroid lines as the sample size). (G–J) Quantitation showing a significantly lower magnitude of forskolin-induced \(\Delta I_{sc}\) in differentiated enteroids compared with undifferentiated enteroids (set as 100%) in (G) KRB buffer, (H) HCO\(_3\)\(^-\)-free buffer, and (I) Cl\(^-\)-free buffer. **\(P < .001\), \(n = 11–19\) experiments with paired enteroid monolayers derived from 4 donors for each buffer condition. (J) Representative time-course changes of forskolin-induced swelling in 3-dimensional duodenal enteroids derived from 2 donors. Forskolin-induced swelling assay was performed as previously reported,\(^{22}\) and in contrast to our previous finding, the current study found greater forskolin-induced swelling in undifferentiated enteroids than differentiated enteroids, which likely resulted from the changes in the composition of expansion medium, with one of the major changes being the removal of nicotanamide.
Figure 6. Effects of selected compounds on TER. Representative traces showing the effects of selective compounds on TER. TER was recorded at the same time as short-circuit current was studied. (A–C) Corresponds to Figure 7A–C, (D) corresponds to Figure 7F, (E and F) corresponds to Figure 8A and B; (G–I) corresponds to Figure 8F–H, (J) corresponds to Figure 9A, and (K and L) corresponds to Figure 9C and D.
component was seen only in KRB buffer that contains HCO$_3^-$/CO$_2$; it was not observed in HCO$_3^-$-free buffer, suggesting that it involves HCO$_3^-$ secretion. In fact, a similar bumetanide-insensitive component also was found in mouse duodenum only when extracellular HCO$_3^-$ was present.$^{31}$ That this was likely to be HCO$_3^-$ secretion was supported further by its sensitivity to acetazolamide, which has been shown to inhibit basal and stimulated HCO$_3^-$ secretion in intact human/animal duodenum.$^{34}$–$^{36}$ Taken together, these results show the similarity of human duodenal enteroid monolayers and intact human/mouse duodenum in many aspects of cAMP-stimulated Cl$^-$ and HCO$_3^-$ secretion.

As we proposed previously,$^{13}$ that forskolin-induced $\Delta I_{sc}$ is detectable in villus-like–differentiated enteroids suggests anion secretion is not strictly confined to crypts, although a significant quantitative difference exists between differentiated and undifferentiated enteroids. The lesser anion secretion in differentiated enteroids seems to be caused largely by the reduction in Cl$^-$ secretion, which is related to the reduced expression of CFTR, NKCC1 and KCNE3, all of which are known to be necessary for cAMP-stimulated Cl$^-$ secretion. Interestingly, several ion transporters and carbonic anhydrase isoforms that are involved in HCO$_3^-$ secretion, including DRA, NBCe1, CA2, and CA4, were increased in differentiated enteroids. These changes may explain that the absolute amount of forskolin-induced $\Delta I_{sc}$ that was inhibited by acetazolamide, which represents a component of HCO$_3^-$ secretion, was similar between undifferentiated and differentiated enteroids despite a significant reduction in CFTR expression in the latter. Further studies are needed to determine whether these changes in expression lead to increased DRA-related Cl$^-$-dependent

---

**Figure 7.** Functional involvement of apical ion transporters in cAMP-stimulated electrogenic anion secretion. (A–C) Representative traces showing the inhibitory effect of CFTRinh-172 (25 μmol/L on the apical side) on forskolin-induced $\Delta I_{sc}$ arising from undifferentiated (UD) and differentiated (DF) enteroid monolayers in (A) KRB buffer, (B) HCO$_3^-$-free buffer, and (C) Cl$^-$-free buffer. (D) Quantitation showing CFTRinh-172 (25 μmol/L on the apical side) inhibited the majority of forskolin-induced $\Delta I_{sc}$ in both undifferentiated and differentiated enteroids in all buffer conditions. $n = 3$–5 experiments with paired enteroid monolayers derived from 2–3 donors for each buffer condition. (E) Quantitation showing a lower dose of CFTRinh-172 (5 μmol/L on the apical side) also inhibited the majority of forskolin-induced $\Delta I_{sc}$ in both undifferentiated and differentiated enteroids in KRB buffer. $n = 3$ experiments with paired enteroid monolayers derived from 2 donors. (F) A representative trace showing the insensitivity of forskolin-induced $\Delta I_{sc}$ to tenapanor in KRB buffer. The cumulative concentration of tenapanor on the apical side after each addition was 0.1 μmol/L, 0.5 μmol/L, and 1 μmol/L. This experiment was repeated at least 3 times using paired enteroid monolayers derived from 2 donors, and similar results were found in each experiment.

2018 Anion Secretion In Human Duodenal Enteroids 605
HCO₃⁻ secretion in differentiated enteroids, a process that was not determined by the Ussing chamber/voltage-current clamp technique used in this study.

The present study allowed further dissection of the contribution of specific transporters to cAMP-stimulated anion secretion in duodenal enteroids. On the apical surface, CFTR was found to be necessary for both Cl⁻ and HCO₃⁻ secretion because CFTRinh-172 abolished the entire forskolin-induced ΔIₑₛ𝑐 in the presence and absence of extracellular Cl⁻ and HCO₃⁻/CO₂ in differentiated as well as undifferentiated enteroids. Thus, CFTR is the major apical transporter for all duodenal cAMP-stimulated anion secretion and no other Cl⁻ channel seems to contribute significantly, unless that contribution is indirect and involves CFTR. Similarly, there appears no role for NHE3 in cAMP-stimulated anion secretion because forskolin-induced ΔIₑₛ𝑐 was not altered by tenapanor. However, NHE3 still could have a role in electroneutral HCO₃⁻ secretion, which could not be quantitated in the current study. Any specific role for DRA and PAT-1 could not be evaluated in this study, although they are likely to contribute to cAMP-stimulated anion secretion through their interaction with CFTR.

In terms of the basolateral transporters participating in cAMP-stimulated Cl⁻ secretion, our results confirmed NKCC1 as the essential basolateral Cl⁻ loader and the necessary contributions of cAMP-activated K⁺ channel(s) and Na⁺/K⁺-ATPase. As for cAMP-stimulated HCO₃⁻ secretion, there are 2 potential pathways that could supply HCO₃⁻ for apical extrusion. One is a Na⁺/HCO₃⁻ co-transporter that interacts with NHE1 to mediate HCO₃⁻ uptake across the basolateral membrane. Our study did not provide any evidence for the involvement of NBCs because a specific inhibitor of the NBC family (S0859) had no significant inhibitory effect but rather a small stimulatory effect on forskolin-induced ΔIₑₛ𝑐. A similar transient stimulatory effect of S0859 on forskolin-induced ΔIₑₛ𝑐 also was seen in bronchial epithelial cells, which could be inhibited by calcium-activated chloride channel (CaCC) inhibitor. The second source of HCO₃⁻ is the production of intracellular HCO₃⁻ by carbonic anhydrase(s), the contribution of which is supported by

**Figure 8. Functional involvement of basolateral ion transporters and carbonic anhydrase(s) in cAMP-stimulated electrogenic anion secretion.** (A and B) Representative traces showing forskolin-induced ΔIₑₛ𝑐 arising from undifferentiated (UD) and differentiated (DF) enteroid monolayers was sensitive to bumetanide (100 μmol/L on the basolateral side) and acetazolamide (250 μmol/L on both apical and basolateral sides) in KRB buffer. The combination of the 2 compounds abolished forskolin-induced ΔIₑₛ𝑐 despite their order. (C and D) Quantitation showing the percentage of forskolin-induced ΔIₑₛ𝑐 that was inhibited by (C) bumetanide and (D) acetazolamide in KRB buffer. Undifferentiated enteroids showed a relatively higher sensitivity to bumetanide than to acetazolamide, whereas differentiated enteroids showed a relatively higher sensitivity to acetazolamide than to bumetanide. The differences between undifferentiated and differentiated enteroids in their sensitivity to bumetanide and acetazolamide were statistically significant (***P < .001). n = 15 experiments with paired enteroid monolayers derived from 4 donors. (E) Quantitation showing the absolute reduction in the magnitude of forskolin-induced ΔIₑₛ𝑐 by acetazolamide in KRB buffer. There was no statistical difference between undifferentiated enteroids and differentiated enteroids. n = 15 experiments with paired enteroid monolayers derived from 4 donors. (F and G) Representative traces showing the effects of ouabain (F, 100 μmol/L on the basolateral side), SITS (G, 1 mmol/L on the basolateral side), and S0859 (H, 30 μmol/L on the basolateral side) on forskolin-induced ΔIₑₛ𝑐 in KRB buffer. These experiments were repeated at least 3 times using paired enteroid monolayers derived from 2 to 3 donors, and similar results were found in each experiment.
the inhibitory effect of acetazolamide on HCO₃⁻ secretion, as shown by multiple studies. In the current study, an acetazolamide-sensitive component was present in cAMP-stimulated anion secretion, indicating the requirement for carbonic anhydrase(s) in HCO₃⁻ secretion. However, the specific carbonic anhydrase isofom was not identified. Two isoforms were up-regulated significantly with differentiation in duodenal enteroids; CA2 is thought to be the major enzyme involved in intracellular HCO₃⁻ production, and CA4 is expressed only in differentiated enteroids and is reported to interact with CFTR and facilitate CO₂ influx.

In conclusion, this study describes some of the molecular basis of cAMP-stimulated anion secretion in normal human duodenal enteroid monolayers and compares the ion transport processes taking part in anion secretion in crypt-like-undifferentiated enteroids and villus-like-differentiated enteroids. We suggest that human enteroid monolayers are a useful tool to study multiple physiological and pathophysiological models of intestinal anion secretion, including the chloride and bicarbonate components, as well as those that are caused separately by crypt-like and villus-like epithelial cells of human small intestine.

References

1. Field M. Intestinal ion transport and the pathophysiology of diarrhea. J Clin Invest 2003;111:931–943.
2. Ikpa PT, Sleddens HF, Steinbrecher KA, Peppelenbosch MP, de Jonge HR, Smits R, Bijvelds MJ. Guanylin and uroguanylin are produced by mouse intestinal epithelial cells of columnar and secretory lineage. Histochem Cell Biol 2016;146:445–455.
3. Ledford H. Translational research: 4 ways to fix the clinical trial. Nature 2011;477:526–528.
4. De Jonge HR. The response of small intestinal villous and crypt epithelium to choleratoxin in rat and guinea pig. Evidence against a specific role of the crypt cells in choleragen-induced secretion. Biochim Biophys Acta 1975;381:128–143.
5. Jakab RL, Collaco AM, Ameen NA. Physiological relevance of cell-specific distribution patterns of CFTR, NKCC1, NBCe1, and NHE3 along the crypt-villus axis in 2018 Anion Secretion In Human Duodenal Enteroids 607

Figure 9. Functional involvement of basolateral transporters in cAMP-stimulated electrogenic Cl⁻ secretion. (A and B) A representative trace and quantitative analysis showing the inhibitory effect of bumetanide (100 μmol/L on the basolateral side) on forskolin-induced ∆Isc arising from undifferentiated (UD) and differentiated (DF) enteroid monolayers in HCO₃⁻-free buffer. n = 4 experiments with paired enteroid monolayers derived from 3 donors. (C) A representative trace showing the inhibitory effect of ouabain (100 μmol/L on the basolateral side) on forskolin-induced ∆Isc in HCO₃⁻-free buffer. This experiment was repeated at least 3 times using paired enteroid monolayers derived from 2 donors, and similar results were found in each experiment. (D and E) A representative trace and quantitative analysis showing the inhibitory effect of chromanol 293B (10 μmol/L on the basolateral side) on forskolin-induced ∆Isc in HCO₃⁻-free buffer. n = 5 experiments with paired enteroid monolayers derived from 2 donors.
the intestine. Am J Physiol Gastrointest Liver Physiol 2011;300:G82–G98.
6. Kockerling A, Fromm M. Origin of cAMP-dependent Cl-
secretion from both crypts and surface epithelia of rat intestine. Am J Physiol 1993;264:C1294–C1301.
7. McNicholas CM, Brown CD, Turnberg LA. Na-K-Cl
cotransport in villus and crypt cells from rat duodenum. Am J Physiol 1994;267:G1004–G1011.
8. Yin J, Tse CM, Cha B, Sarker R, Zhu XC, Walentinsson A,
Greasley PJ, Donowitz M. A common NHE3 single
nucleotide polymorphism has normal function and
sensitivity to regulatory ligands. Am J Physiol Gastro-
intest Liver Physiol 2017;313:G129–G137.
9. Foulke-Abel J, In J, Yin J, Zachos NC, Kovbasnjuk O,
Estes MK, de Jonge H, Donowitz M. Human enteroids
as a model of upper small intestinal ion transport
physiology and pathophysiology. Gastroenterology 2016;
150:638–649 e8.
10. Stewart CP, Turnberg LA. A microelectrode study of
responses to secretagogues by epithelial cells on villus and
crypt of rat small intestine. Am J Physiol 1989;
257:G334–G343.
11. Sato T, Stange DE, Ferrante M, Vries RG, Van Es JH, Van
den Brink S, Van Houdt WJ, Pronk A, Van Gorp J,
Siersema PD, Van Houdt WJ, Pronk A, Van Gorp J,
Hepatol 2017;3:422.
12. Sato T, Vries RG, Snippert HJ, van de Wetering M,
Barker N, Stange DE, van Es JH, Abo A, Kujala P,
Peters PJ, Clevers H. Single Lgr5 stem cells build crypt-
villus structures in vitro without a mesenchymal niche.
Nature 2009;459:262.
13. Yu H, Hasan NM, In JG, Estes MK, Brown CD, Turnberg LA. Na-K-Cl
cotransport in villus and crypt cells from rat duodenum. Am J Physiol 1994;267:G1004–G1011.
14. Ettayebi K, Crawford SE, Murakami K, Broughman JR,
Karandikar U, Tenge VR, Neill FH, Blutt SE, Zeng XL,
Qu L, Kou B, Opekun AR, Burrin D, Graham DY, Ramani S, Atmar RL, Estes MK. Replication of human
noroviruses in stem cell-derived human enteroids.
Science 2016;353:1387–1393.
15. Noel G, Baetz NW, Staab JF, Donowitz M, Kovbasnjuk O,
Pasetti MF, Zachos NC. A primary human macrophage-
enteroend co-culture model to study mucosal gut
physiology and host-pathogen interactions. Sci Rep
2017;7:45270.
16. In J, Foulke-Abel J, Zachos NC, Hansen AM, Kaper JB,
Bernstein HD, Halushka M, Blutt S, Estes MK,
Donowitz M, Kovbasnjuk O. Enterohemorrhagic reduce
mucous and intermicrovillar bridges in human stem cell-
derived colonoids. Cell Mol Gastroenterol Hepatol
2016;2:48–62 e3.
17. Sato T, Clevers H. Primary mouse small intestinal
testinal epithelial cell cultures. Methods Mol Biol 2013;
945:319–328.
18. Heijmans J, van Lidde de Jeude JF, Koo BK, Rosekrans SL,
Wielenga MC, van de Wetering M, Ferrante M, Lee AS, Onderwater JJ, Paton JC,
Paton AW, Mommaas AM, Kodach LL, Hardwick JG,
Hommes DW, Clevers H, Muncan V, van den Brink GR.
ER stress causes rapid loss of intestinal epithelial
stemness through activation of the unfolded protein
response. Cell Rep 2013;3:1128–1139.
19. Clarke LL. A guide to Ussing chamber studies of mouse
intestine. Am J Physiol Gastrointest Liver Physiol 2009;
296:G1151–G1166.
20. Thompson CA, Wojta K, Pulakanti K, Rao S, Dawson P,
Battle MA. GATA4 is sufficient to establish jejunal versus
ileal identity in the small intestine. Cell Mol Gastroenterol
Hepatol 2017;3:422–446.
21. Middendorp S, Schneeberger K, Wiegerinck CL,
Mokry M, Akkerman RD, van Wijngaarden S, Clevers H,
Nieuwenhuis EE. Adult stem cells in the small intestine
are intrinsically programmed with their location-specific
function. Stem Cells 2014;32:1083–1091.
22. Fuji M, Matano M, Nanki K, Sato T. Efficient genetic
engineering of human intestinal organoids using elec-
etropermeation. Nat Protoc 2015;10:1474–1485.
23. Tang L, Fatehi M, Linsdell P. Mechanism of direct
bicarbonate transport by the CFTR anion channel. J Cyst
Fibros 2009:8:115–121.
24. Cil O, Phuan PW, Gillespie AM, Lee S, Tradrantlip L,
Yin J, Tse M, Zachos NC, Lin R, Donowitz M,
Verkman AS. Benzopyrido-pyrrolo-oxazine-dione
CFTR inhibitor (R)-BPO-27 for antisecretory therapy of
diarreas caused by bacterial enterotoxins. FASEB J
2017;31:751–760.
25. Gawenis LR, Bradford EM, Alper SL, Prasad V, Shull GE.
AE2 Cl-/-HCO3- exchanger is required for normal cAMP-
stimulated anion secretion in murine proximal colon. Am J
Physiol Gastrointest Liver Physiol 2010; 298:G493–G503.
26. Ch’en FF, Villafuerte FC, Swietach P, Cobden PM,
Vaughan-Jones RD. S0859, an N-cyanosulphonamide
inhibitor of sodium-bicarbonate cotransport in the heart.
Br J Pharmacol 2008;153:972–982.
27. Mall M, Kunzelmann K, Hipper A, Busch AE, Greger R.
cAMP stimulation of CFTR-expressing Xenopus oocytes
activates a chromanol-inhibitable K+ conductance.
Pflugers Arch 1996;432:516–522.
28. Singh AK, Riederer B, Chen M, Xiao F, Krabbenhöft A,
Engelhardt R, Nylander O, Soleimani M, Seidler U. The
switch of intestinal Slc26 exchangers from anion
absorptive to HCO3- secretory mode is dependent on
CFTR anion channel function. Am J Physiol Cell Physiol
2010;298:C1057–C1065.
29. Shan J, Liao J, Huang J, Robert R, Palmer ML,
Fahrenkrug SC, O’Grady SM, Hanrahan JW. Bicarbonate-
dependent chloride transport drives fluid secretion by the
human airway epithelial cell line Calu-3. J Physiol 2012;
590:5273–5297.
30. Hong JH, Yang D, Shcheynikov N, Ohana E, Shin DM,
Muallem S. Convergence of IRBIT, phosphatidylinositol
(4,5) bisphosphate, and WNK/SPAK kinases in regulation
of the Na+–HCO3- cotransporters family. Proc Natl Acad
Sci U S A 2013;110:4105–4110.
31. Clarke LL, Stien X, Walker NM. Intestinal bicarbonate
secretion in cystic fibrosis mice. JOP 2001;2:263–267.
32. Seidler U, Blumenstein I, Kretz A, Viellard-Baron D, Rossmann H, Colledge WH, Evans M, Ratcliff R, Gregor M. A functional CFTR protein is required for mouse intestinal cAMP-, cGMP- and Ca2+−dependent HCO3- secretion. J Physiol 1997;505:411–423.

33. Walker NM, Flagella M, Gawenis LR, Shull GE, Clarke LL. An alternate pathway of cAMP-stimulated Cl- secretion across the NKCC1-null murine duodenum. Gastroenterology 2002;123:531–541.

34. Knutson TW, Koss MA, Hogan DL, Isenberg JI, Knutson L. Acetazolamide inhibits basal and stimulated HCO3- secretion in the human proximal duodenum. Gastroenterology 1995;108:102–107.

35. Muallem R, Reimer R, Odes HS, Schwenk M, Beil W, Sewing KF. Role of carbonic anhydrase in basal and stimulated bicarbonate secretion by the guinea pig duodenum. Dig Dis Sci 1994;39:1078–1084.

36. Jacob P, Christiani S, Rossmann H, Lamprecht G, Viellard-Baron D, Müller R, Gregor M, Seidler U. Role of Na+/HCO3- cotransporter NBC1, Na+/H+ exchanger NHE1, and carbonic anhydrase in rabbit duodenal bicarbonate secretion. Gastroenterology 2000;119:406–419.

37. Walker NM, Simpson JE, Brazil JM, Gill RK, Dudeja PK, Schweinfest CW, Clarke LL. Role of down-regulated adenoma anion exchanger in HCO3- secretion across murine duodenum. Gastroenterology 2009;136:893–901.

38. Preston P, Wartosch L, Günzel D, Fromm M, Kongsuphol P, Ousingsawat J, Kunzelmann K, Barhanin J, Warth R, Jentsch TJ. Disruption of the K+ channel beta-subunit KCNE3 reveals an important role in intestinal and tracheal Cl- transport. J Biol Chem 2010;285:7165–7175.

39. Seidler U, Bachmann O, Jacob P, Christiani S, Blumenstein I, Rossmann H. Na+/HCO3- cotransport in normal and cystic fibrosis intestine. JOP 2001;2:247–256.

40. Gorrieri G, Scudieri P, Caci E, Schiavon M, Tomati V, Sirchi F, Napolitano F, Carrella D, Gianotti A, Musante I, Favia M, Casavola V, Guerra L, Rea F, Ravazzolo R, Di Bernardo D, Galletti LJ. Goblet cell hyperplasia requires high bicarbonate transport to support mucin release. Sci Rep 2016;6:36016.

41. Fanjul M, Salvador C, Alvarez L, Cantet S, Hollande E. Targeting of carbonic anhydrase IV to plasma membranes is altered in cultured human pancreatic duct cells expressing a mutated (ΔF508) CFTR. Eur J Cell Biol 2002;81:437–447.

42. Musa-Aziz R, Occhipinti R, Boron WF. Evidence from simultaneous intracellular- and surface-pH transients that carbonic anhydrase IV enhances CO2 fluxes across Xenopus oocyte plasma membranes. Am J Physiol Cell Physiol 2014;307:C814–C840.