Chapter 1
Organoids as a Model for Intestinal Ion Transport Physiology

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Abstract The advent of intestinal organoid culture in 2009 was a fortuitous development in the search for a valid marker of intestinal stem cells, and provided proof of murine intestinal stem cell regenerative potential. Intestinal organoid culture was preceded by key discoveries of the Wnt/β-catenin signaling pathway and the development of 3D culture matrices. The latter, involving a laminin-rich gel to provide an artificial basement membrane, was instrumental to primary intestinal epithelial culture by preventing anoikis, an immediate apoptotic event when intestinal epithelial cells detach from the basement membrane. One of the first physiological studies using 3D murine "mini-gut" structures showed cystic fibrosis transmembrane conductance regulator (CFTR) expression and anion channel activity in the crypt-like structures projecting from the epithelial-lined central cavity. Detailed investigations of ion transport physiology using human intestinal organoids, both primary and iPSC-derived, found close similarities to existing knowledge of ion transport physiology and included the development of the forskolin-induced swelling assay (FIS). The FIS assay using organoids cultured from rectal biopsies of cystic fibrosis patients provided an avenue for personalized medicine to test small-molecule modulators on different CFTR mutations. More recent research has led to the development of 2D primary intestinal epithelial monolayers, which provide easy access to the apical, lumen-facing membrane and the opportunity for traditional ion transport studies with Ussing chambers. Human 2D primary intestinal monolayers also demonstrate the dominance of CFTR in anion secretion and provide a quantitative

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evaluation of its chloride and bicarbonate secretory conductances. These aspects of ion transport physiology using 2D and 3D intestinal cultures are discussed along with the relative advantages and disadvantages of each culture method with respect to technical aspects and recapitulation of native intestinal epithelium.

Keywords Organoid · Enteroid · Colonoid · Intestine · Colon · CFTR · Cystic fibrosis · Precision medicine · Personalized medicine · Forskolin-induced swelling · Human · Mouse

1.1 Introduction

The culture of self-renewing primary intestinal organoids is one of the most significant experimental techniques developed for investigation of intestinal ion transport physiology. Before 2009, most research of ion transport physiology in intestinal epithelia involved studies of the whole animal (in vivo), short-term tissue cultures or intestinal cell lines, the latter enabling cell-based experiments and genetic manipulations. Studies of the native crypt, i.e., the stem and progenitor cell compartment, were particularly difficult because of the morphological structure and limited access in the intestine in vivo and ex vivo. Attempts at a regenerating primary culture were largely unsuccessful, with a few heroic exceptions (reviewed in Evans et al. 1994). In retrospect, it may be surmised that the technical difficulties were largely a consequence of the propensity of intestinal epithelia to undergo anoikis—an apoptotic event triggered when the epithelial cell detaches from the basement membrane. Short-term studies of isolated colonic crypts were possible (Robert et al. 2001; Singh et al. 1995; Greger et al. 1997; Reynolds et al. 2007; Mignen et al. 2000), apparently due to a higher resistance to anoikis, and provided important insight into the ion transport physiology of the crypt epithelium. These investigations were followed by the pioneering studies of Williams et al. in 2007 that extended the utility of isolated colonic crypts to several days by the provision of appropriate culture substrates (Reynolds et al. 2007). Around the turn of the century, a growing recognition of the importance of the interplay between the epithelium and the extracellular matrix leading to the development of 3D gel cultures in other epithelial cells set the stage for successful primary organoid culture of murine intestinal epithelium (Hofmann et al. 2007; Ootani et al. 2009). A breakthrough came with the recognition of R-spondin1 as an important mitogenic intestinal growth factor that was eventually found to be a secreted coagonist of Wnt/β-catenin signaling in intestinal stem cells (ISCs) (Kim et al. 2005). Using R-spondin1-supplemented medium, a robust, regenerating intestinal culture was developed using minced mouse intestine that gave rise to cysts in a 3D collagen gel (Ootani et al. 2009). The cysts were composed of a polarized, quasidifferentiated intestinal epithelium with crypt- and occasional villus-like structures in a surrounding layer of mesenchymal cells.

The development of a self-renewing, pure epithelial culture came from the search for unique markers of intestinal stem cells. Hans Clevers' laboratory reported the discovery of a Wnt target gene, leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5) that was expressed in cycling crypt-base columnar cells, i.e., cells originally described with potential for stem cell activity (Cheng and Leblond 1974; Barker et al. 2007). Lineage tracing experiments using mice expressing the knock-in alleles Lgr5-IRES-LacZ or the fusion Lgr5-EGFP-IRES-Cre-ERT2 showed that Lgr5+ cells generated all intestinal lineages, actively cycled and were located at both the crypt base (90%) and at the +4 cell position (10%), i.e., the locale of long-term label-retaining cells that are now referred to as quiescent stem cells (Barker et al. 2007). In 2009, Clevers' group provided positive verification of stem cell status for Lgr5+ cells and introduced the culture of well-differentiated, intestinal epithelial organoids to the field of gastroenterology research. In this study (Sato et al. 2009), a single Lgr5-GFP expressing cell from the Lgr5–EGFP–ires–CreERT2 small intestine was isolated by cell sorting and plated in laminin-rich Matrigel® in medium containing the Wnt signaling cofactor Rspondin1, epidermal growth factor (EGF), noggin (a bone morphogenic protein inhibitor), and 20% FBS. Over a 2-week period, the cells multiplied and gave rise to an intestinal epithelial organoid with a central cavity composed of differentiated cells (i.e., the villus-like domain) from which were outgrowths of organotypic crypts with the actively recycling Lgr5-positive stem cells localized at the crypt base. Fortunately, the isolation of individual Lgr5-positive stem cells was not necessary to culture mouse small intestinal organoids (enteroids, Stelzner et al. 2012) in that freshly isolated small intestinal crypts could be cultured in the same manner to form multiple intestinal epithelial organoids.

One of the favorable features of the enteroid model that was immediately apparent was the visual access provided by the 3D gel culture. Overcoming the difficulties of visualizing crypt epithelium in vivo and the onset of anoikis in epithelial cells of isolated crypts, the enteroid model enabled observations of individual epithelial cell types within the context of a model of native intestine. Evaluation of the crypt epithelium of the enteroid indicated polarization as denoted by an apical microvillus brush border that increased in length along cells in the upper crypt. Further, all the major cell lineages of the small intestine were represented, including absorptive enterocytes, goblet cells, enteroendocrine cells and Paneth cells in approximately the same percentages as found in the intestine in vivo (Sato et al. 2009). The organoids were devoid of nonepithelial cell types, could be passaged at weekly intervals for extended times (months), maintained euploidy and demonstrated a gene expression profile similar to freshly isolated crypts. Shortly after these studies, investigation of the fluorescent signature of crypt-base cells from mice expressing eGFP-labeled Sox9, a transcription factor enriched in Lgr5+ ISCs, recapitulated evidence that a single stem cell could generate differentiated organoids (Gracz et al. 2010). Subsequent analysis of the requirements for enteroid production led to the discovery that single, isolated Lgr5-positive stem cells have a low capacity to develop into enteroids (5%), whereas cell doublets of Lgr5-positive stem cells and lysozyme-positive Paneth cells have a high capacity (80%) for the

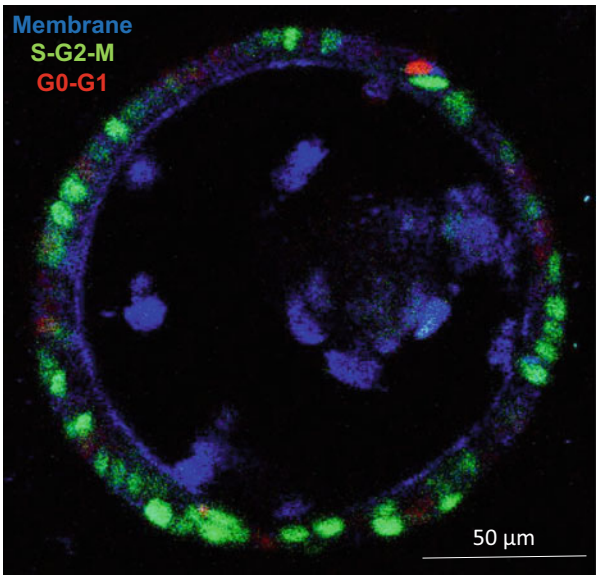

Fig. 1.1 FUCCI2 enterosphere. Optical cross-section of an intestinal stem cell-enriched enterosphere from a Fucci2 mouse intestine (Matsu-Ura et al. 2016), provided by RIKEN Center for Life Science Technologies (Kobe). Cell nuclei indicate quiescent cells (G0–G1 phases, red nuclei) and actively cycling cells (S-G2-M phases, green nuclei). Enterosphere cultured in Matrigel using a modified Sato method (Liu et al. 2012) and supplemented with Wnt3a 100 ng/mL for 48 h

development of enteroid structures (Sato et al. 2011b). Indeed, isolated Lgr5 + ISCs and Paneth cells mixed together in 3D gel culture avidly seek cell-to-cell contact between the two cell types by an, as yet, undescribed process of cell recognition and homing. A key feature of the enteroid model is the elaboration of Wnt3a by Paneth cells, which together with the addition of the Lrg5 ligand Rspondin-1, provides the continual self-renewal of primary enteroids and enables experiments of longer duration, along with the opportunity for genetic manipulations (Schwank et al. 2013).

The enteroid model system of both mouse and human intestine recapitulates many features of small intestinal specialization along the cephalocaudal axis, which is controlled by several transcription factors, in particular GATA4 that is expressed in the proximal intestine and suppresses the expression of distal-specific genes, e.g., the bile-acid transporter ASBT (Slc10a2) (VanDussen et al. 2015; Middendorp et al. 2014). Intestinal organoids also differ regionally with regard to the production of epithelial-autonomous Wnt in that enteroids from the terminal ileum proliferate and develop at slower rates than their more proximal counterparts. Organoids from large intestinal epithelium (aka colonoids) require exogenous Wnt supplementation for growth and development (Yui et al. 2012). Provision of supplemental Wnt ligand or Wnt agonists (e.g., CHIR 99021, an inhibitor of GSK 3β) to small intestinal enteroids also enhances proliferation and suppresses differentiation leading to the expansion of the ISC population (also termed undifferentiated cells) to form “enterospheres” that are composed of a single cell layer in a spheroid structure. As shown in Fig. 1.1, ISC-enriched enterospheres have a majority of cells in active stages of the cell cycle as shown by enterospheres from Fucci2 reporter mouse intestine. Together, these advances have enabled the expansion of intestinal

epithelium from patients with intestinal disease that are proving to be valuable for translational studies and individualized medicine in gastroenterology (VanDussen et al. 2015; Sato et al. 2011a).

1.2 Ion Transport in 3D Mouse Intestinal Organoids

The earliest studies of ion transport physiology in the enteroid model were driven by an evaluation of the cystic fibrosis transmembrane conductance regulator anion channel CFTR (Liu et al. 2012). CFTR is the protein product of the gene that is mutated in the monogenic disease cystic fibrosis (CF), the most common lethal genetic mutation of people from a northern European background (Collins 1992). CFTR channel activity is the principal pathway for the secretion of Cl and HCO_3^- across the intestinal epithelium as well as the respiratory and pancreatic duct epithelia (Quinton 1999). Loss of CFTR function results in the dehydration of mucus secreted onto the epithelial surface, causing the accretion of abnormally viscous mucus, a condition known as mucoviscidosis. This pathogenic process underlies most CF disease manifestations, including the failure of mucociliary clearance from airways, intestinal impaction/constipation and plugging of pancreatic ducts with the sequela of pancreatic insufficiency. In contrast to CF, protracted hyperactivation of CFTR by microbial toxins strongly enhances fecal loss of salt and water to produce systemic dehydration and acidosis. Such "secretory" diarrheas typically ensue from colonization of the gut by enterotoxigenic bacteria, including Vibrio cholera (causing cholera) and specific *Escherichia coli* strains (causing colibacillosis, e.g., Traveler's diarrhea) (Barrett and Keely 2000).

CFTR activity in intestinal organoids was first assessed by comparing expression and functional responses in enteroids from wild-type (WT) and Cftr knockout (Cftr KO) mice (Liu et al. 2012). Using an adaptation of the culture method of Sato et al. (2011a), Liu and colleagues showed Cftr protein expression in passaged WT enteroids that was comparable in magnitude to expression in freshly isolated WT crypts and absent in enteroids from sex-matched littermate Cftr KO mice. Microelectrode analysis of Cftr function by impalements of crypt base epithelial cells was possible by gentle aspiration of the encasing Matrigel® via a micropipette to expose the basolateral side of the epithelium in enteroid crypts (Fig. 1.2a). As shown in Fig. 1.2b, c, a basolateral membrane potential of −40 mV measured in WT enteroid crypt epithelial cells abruptly depolarizes upon exposure to forskolin, a cyclic AMP agonist used to stimulate Cftr activity. The inward current produced by stimulation of the anion conductance was Cftr-dependent as shown by the failure of Cftr KO crypt epithelial cells to depolarize. Further, WT crypt epithelia exhibited partial repolarization of forskolin-stimulated enteroids upon acute treatment with the CFTR inhibitor $CFTR_{inh}172$ (Fig. 1.2d).

A second feature of Cftr function investigated in the mouse enteroid model was regulation of intracellular pH (pH_i) in the crypt epithelium. CFTR is conductive to both Cl^- and HCO_3^- anions with a relative permeability of ~4:1, respectively

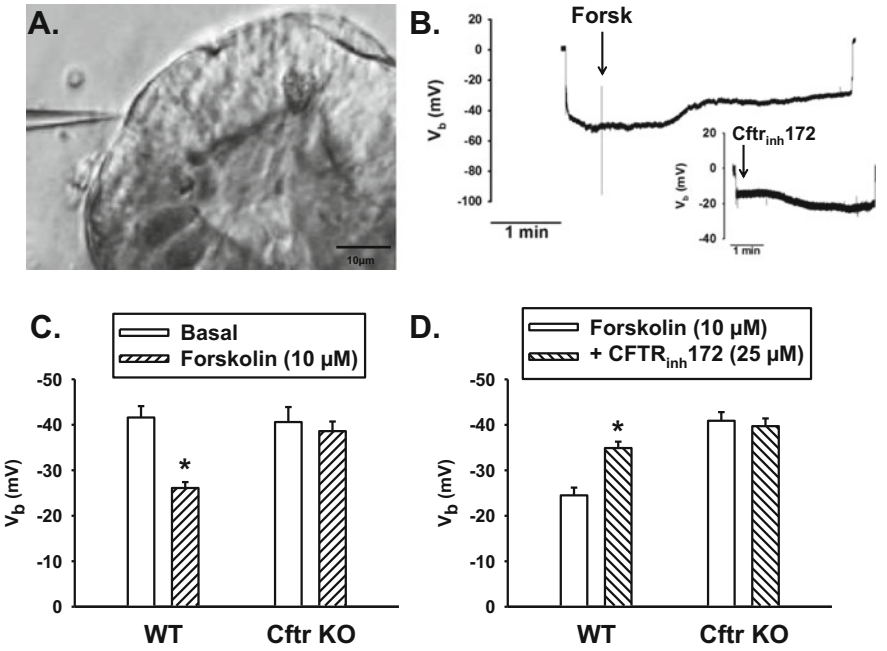

Fig. 1.2 Microelectrode analysis of Cftr-dependent changes in membrane potential in enteroid crypt epithelium. (**a**) Micrograph of enteroid crypt epithelial cell impaled with a conventional microelectrode (magnification: ×200). (**b**) Representative recording of the basolateral membrane potential (V_b) of an impaled WT cell before and after exposure to 10 μM forskolin. *Inset*: representative recording of V_b in an impaled cell from the same forskolin-treated WT enteroid crypt before and after exposure to 25 μM $Cftr_{inh}172$. Note abrupt voltage change from 0 mV upon microelectrode impalement and return toward 0 mV upon microelectrode retraction in both recordings. However, there was a ~4-mV electrode drift during the prolonged impalement showing forskolin-induced depolarization. (**c**) Cumulative data of mean V_b measured in WT and Cftr KO enteroid crypt epithelial cells before (basal) and after exposure to 10 μM forskolin. Impalements during forskolin were performed 10–30 min after treatment. Enteroids were from WT and Cftr KO sex-matched littermate mice. $^*P < 0.05$, significantly different from basal within genotype; $n = 33$–34 impalements, 4–6 p0–p1 enteroids from 3 mice pairs. Mean V_b for Cftr KO, both basal and forskolin-treated, is significantly greater than WT treated with forskolin, $P < 0.05$. (**d**) Cumulative data of mean V_b measured in WT and Cftr KO forskolin-treated enteroid crypt epithelial cells before (forskolin: 10 μM) and after exposure to 25 μM $Cftr_{inh}172$. Enteroids were exposed to forskolin for 10 min before microelectrode impalements. Impalements during $Cftr_{inh}172$ were performed 10–30 min after treatment. Enteroids were from WT and Cftr KO sex-matched littermate mice. $^*P < 0.05$, significantly different from forskolin within genotype; $n = 16$–26 impalements, 3–4 p0–p1 enteroids from 3 mice pairs (Liu et al. 2012)

(Poulsen et al. 1994). CFTR also facilitates HCO_3^- secretion by directly enhancing apical membrane Cl^-/HCO_3^- exchanger activity, notably Slc26a3 and Slc26a6 anion exchangers (Dorwart et al. 2008), by providing Cl^- recycling that prevents the development of an unfavorable inside to outside Cl^- concentration gradient for the exchange process (Simpson et al. 2005). Moreover, Slc26a9, a complex anion

transporter whose function is CFTR-dependent in airway epithelium (Bertrand et al. 2017), has been shown to provide HCO_3^- secretion from a crypt-predominant location in the proximal duodenum (Liu et al. 2014). Thus, loss of CFTR, as a major pathway for HCO_3^- efflux from intestinal epithelial cells, sets the stage for pH_i dysregulation. Previous studies of cell lines and mouse intestinal villi have shown that CFTR expression/activity physiologically acidifies pH_i and, in its absence, cells exhibit an alkaline pH_i (Barriere et al. 2001; Elgavish 1991; Gottlieb and Dosanjh 1996; Simpson et al. 2005; Hirokawa et al. 2004). In accordance with those studies, Liu et al. (2012) found that the crypt epithelium of Cftr KO enteroids exhibited an alkaline pH (~7.5) relative to crypts from WT littermate enteroids (~7.2). WT crypts also acutely alkalized upon treatment with $CFTR_{inh}172$ (Fig. 1.3a, c). The observation that the alkaline pH_i in the Cftr KO crypt epithelium was not normalized by the activity of other acid-base transport processes led to further investigations using mouse enteroids. Walker et al. showed that the expression of anion exchanger 2 (AE2) is upregulated in Cftr KO enteroids, whereas the expression of Slc26a3, the Na^+/H^+ exchanger Nhe2, and the transmembrane carbonic anhydrase CA9 is decreased—all appropriate changes to compensate for an alkaline pH_i (Walker et al. 2016). However, the Cl^-/HCO_3^- exchange activity of Ae2 was reduced in the Cftr KO crypts due to coincident increases of intracellular Cl^-. Chloride retention in the Cftr KO mouse intestine is a finding that is consistent with previous X-ray microprobe analysis of human CF intestine (O'Loughlin et al. 1996). Chloride retention establishes an unfavorable $[Cl^-]_{in}$ to $[Cl^-]_{out}$ gradient, which retards the exchange process by Ae2 in Cftr KO crypt epithelium. Of note, a technical problem encountered in these studies was persistent retention of the Cl^- sensitive fluorescent dye MQAE within the Matrigel®, despite numerous washings. To overcome this obstacle Walker et al. removed the enteroids from Matrigel®, stabilizing the enteroids with a holding micropipette and included LY2763, a cell-permeant anoikis inhibitor, in the superfusate during the experiment.

The third piece of evidence for functional Cftr activity in the enteroid model is the role that Cftr plays in cell volume regulation. Earlier studies established that activation of CFTR reduces intestinal crypt epithelial cell volume (Valverde et al. 1995; MacLeod et al. 1994), which extends to villi of the duodenum that also express significant levels of CFTR (Gawenis et al. 2003; Strong et al. 1994). The volume reduction in villi also reduces NaCl absorption across villi by downregulating the activity of Na^+/H^+ exchanger isoform 3 (NHE3) (Gawenis et al. 2003; Szászi et al. 2001; Kapus et al. 1994), thereby contributing to the cAMP-induced inhibition of NHE3 mediated by the NHE regulatory protein NHERF (Avula et al. 2018; Seidler et al. 2009). Stimulation of CFTR reduces epithelial cell volume primarily by decreasing the intracellular Cl^- concentration as demonstrated in the elegant studies performed by Foskett and colleagues on airway serous gland epithelial cells (Lee and Foskett 2010; Foskett 1990). In the enteroid study, Liu et al. show that forskolin stimulation in WT enteroids causes a sustained decrease in the enteroid cell volume (~−25%), as indexed by the change in epithelial cell height, a response that is absent in the Cftr KO enteroid crypts (Fig. 1.4a, b) (Liu et al. 2012). Subsequent treatment of WT enteroids with $CFTR_{inh}172$ significantly reduced the cell shrinkage. Although

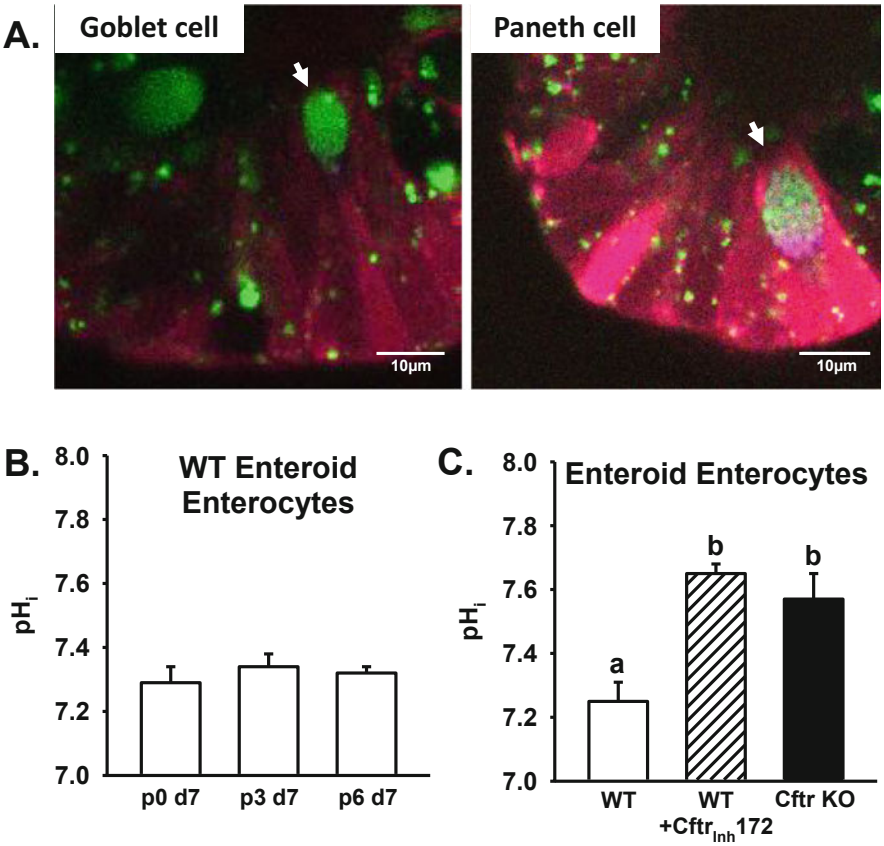

Fig. 1.3 Cftr-dependent effect on basal pH_i in enteroid crypt epithelium. (**a**) Merged confocal images of enteroid crypt epithelium stained with quinicrine (green) to identify granulated secretory cells (goblet and Paneth) and the pH-sensitive dye SNARF 5F (red) for measurement of pH_i (magnification: ×630, n.a. 1.2). (**b**) Mean enterocyte pH_i measured in p0, p3, and p6 enteroids after *day 7* (d7) in culture. Each group of enteroids were from the same WT mice ($n = 3$). (**c**) Mean enterocyte pH_i measured in WT, WT pretreated for 1 h with 25 μM $Cftr_{inh}172$ (WT + Cftrinh172) and Cftr KO enteroid crypts. Enteroids (p0–p1) were from WT and Cftr KO sex-matched littermate mice. $^{a,b}P < 0.05$, means with the same letter are not significantly different; $n = 6$ mouse pairs (Liu et al. 2012)

briefly mentioned, a technical difficulty encountered in studies of cell volume regulation was that WT enteroids would rapidly swell upon forskolin stimulation as a consequence of the luminally directed Cftr-mediated fluid secretion and reduced paracellular fluid leakage through well-developed tight junctions. This combination in the WT enteroid generated sufficient backpressure to flatten the epithelium uniformly, thereby obviating the measurements of cell volume after forskolin. Although enteroid swelling would later become the basis for screening CFTR modulator drugs (see Sect. 1.4, below), the enteroids in the studies by Liu et al.

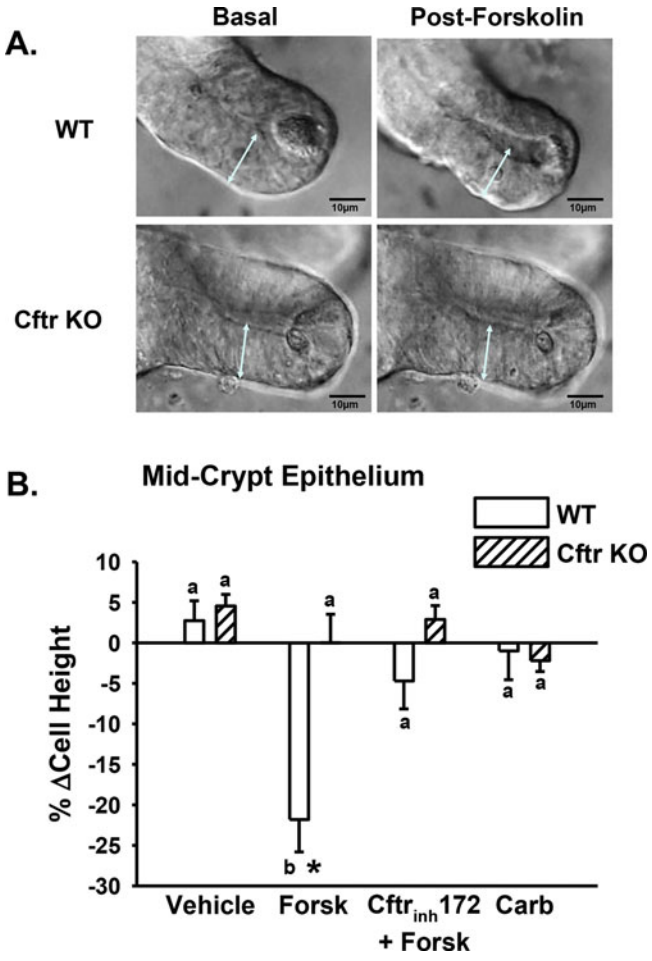

Fig. 1.4 Cftr-dependent cell shrinkage in enteroid crypts. (**a**) Photomicrographs of WT and Cftr KO enteroid crypts before (basal) and after 10-min exposure to 10 μM forskolin (post-forskolin). Arrowed bar indicates measurement of epithelial cell height as an index of cell shrinkage before forskolin treatment. Dotted white and solid white lines indicate diameters (*2r*) of crypt and crypt lumen, respectively, and dashed white line indicates height (*h*) for calculation of epithelial volume before forskolin treatment (magnification: ×400). Enteroids were from WT and Cftr KO sex-matched littermate mice. Mean cell height before treatments were WT = 21.1 ± 0.9 μm and Cftr KO = 21.3 ± 0.9 μm; $n = 18$. (**b**) Cumulative data of % change in epithelial volume after vehicle (Vehicle), forskolin (Forsk, 10 μM), pretreatment with 25 μM $Cftr_{inh}172$ + forskolin ($Cftr_{inh}172$ + Forsk), or carbachol (Carb, 100 μM) in midcrypt epithelium (between position +8 to +15) of paired WT and Cftr KO enteroids (p0–p1). Epithelial volume was calculated by subtracting the crypt luminal volume from the total crypt volume between cell positions +8 to +15, assuming each with a cylindrical shape, using the formula $\pi \cdot h \cdot r^2$ and averaged measurements of the height (*h*) and radius (*r*) of each in optical cross sections. %ΔEpithelial volume was calculated from the formula: change in epithelial volume (in μm^3)/basal epithelial volume (in μm^3) * 100. [a,b]Means with the same letter are not significantly different within genotype. *$P < 0.05$, significantly different from Cftr KO; $n = 6$ WT and Cftr paired enteroids (Liu et al. 2012)

were bisected manually before forskolin exposure to prevent enteroid swelling and, thereby, ensure the accuracy of the epithelial cell volume measurements during forskolin stimulation.

Soon after these studies, Engevik and others used the mouse enteroid model to evaluate the effects of electroneutral NaCl absorption on the microbiota of the distal intestine (Engevik et al. 2013). These investigators showed that Nhe3 knockout mice (Nhe3−/−) have increased intestinal lumen Na^+ concentration and pH, a consequence of lacking the Na^+/H^+ exchange activity at the luminal membrane. Nhe3−/− mice also had alterations in the composition of the microbiota in the distal intestine including increases in Bacteroidetes spp. and an increase in *fut2* expression causing surface fucosylation. To show that the abnormal increase in surface fucosylation was due to the altered microbiota and not a consequence of epithelial Nhe3 ablation per se, these investigators used Nhe3−/− ileal enteroids and found they did not develop surface fucosylation spontaneously, but did so after intraluminal injection of Bacteroidetes spp. into the enteroids. Although ion transport studies of the enteroids were not performed, this research showed two important aspects of the utility of intestinal organoids. First, it demonstrated the potential to investigate host-microbe interactions in organoids with regard to ion transport. Secondly, it demonstrated the opportunity to separate epithelial-autonomous functions such as ion transport from the influences of the intestinal environment, i.e., microbiota and aspects of humoral, neural, submucosal, and immunological regulation.

As mentioned in reference to Fig. 1.1, stem cell proliferation in enteroids can be greatly enhanced through treatment with Wnt3a supplementation resulting in the generation of ISC-enriched "enterospheres or enterospheroids" (Miyoshi and Stappenbeck 2013; Miyoshi et al. 2012). Using the enterosphere model, Strubberg et al. showed that the hyperproliferative state previously demonstrated in the intestinal epithelium of Cftr KO mice in vivo extended to the Cftr KO ISC population (Strubberg et al. 2018; Gallagher and Gottlieb 2001). Further evidence that Wnt3a supplementation yields a model for ISC investigation can be demonstrated in developed enteroids (4 days old) using a single dose of Wnt3a (100 ng/mL). As shown in Fig. 1.5, Wnt3a supplementation causes enteroids to assume an enlarged spheroid shape that, in time, gives rise to an extraordinary increase in crypt structures, an index of ISC proliferation (Fuller et al. 2012). The enterosphere model also demonstrates an important role for Cftr in regulating proliferation of ISCs. Fresh enterospheres from WT and Cftr KO mice generated in the presence of Wnt3a supplementation exhibit a marked difference in luminal volume (as indexed by spheroid diameter) and cell height (Fig. 1.6a), similar to that demonstrated previously in mouse colonoids treated with Wnt3a (Dekkers et al. 2013). Forskolin stimulation causes a rapid increase of sphere diameter in 100% of WT mouse enterospheres and 0% in Cftr KO enterospheres, indicating the presence of functional Cftr in these ISC-enriched structures (Fig. 1.6b, c). The demonstration of functional Cftr activity at an early stage of enterosphere development (2 days) lends support to previous evidence that CFTR is a target of Wnt signaling through an intestine-specific enhancer element located within the first intron (Paul et al. 2007). However, as shown in Fig. 1.6d, e, acute treatment of Wnt3a-treated WT

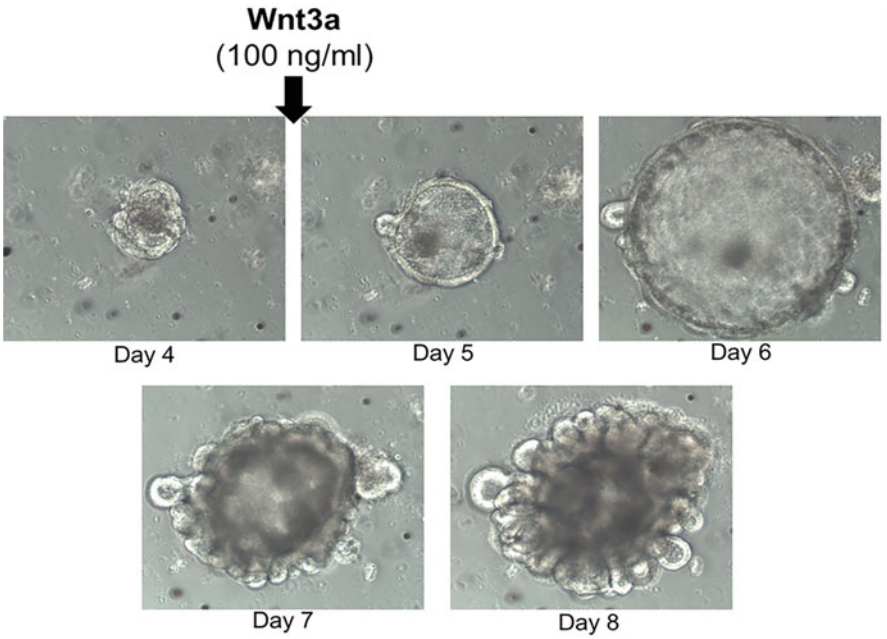

Fig. 1.5 Time course of WT enteroid treated with a single dose of Wnt3a (100 ng/mL on Day 4 in culture). Note expansion of organoid after 2 days (Day 6) and subsequent formation of multiple crypts (Day 8)

enterospheres to inhibit Cftr-mediated anion secretion either by blocking Cl^- uptake by $Na^+/K^+/2Cl^-$ cotransport with bumetanide or by treatment with $CFTR_{inh}$-172, caused a reduction in enterosphere diameter in only a fraction of the structures (30–35%). This observation requires further investigation in that it may reflect limitations in drug delivery through the Matrigel® for certain compounds or, may indicate changes to the Cftr-dependent anion secretory process when enterospheres attain a specific level of turgor or membrane stretch (see Sect. 1.4).

1.3 Ion Transport in 3D Human Intestinal Organoids

The next steps in the investigation of ion transport physiology using intestinal organoids came with the developments of intestinal-differentiated induced pluripotent stem cells (gut-iPSCs) and primary organoid culture of human intestinal epithelium (Sato et al. 2011a; Spence et al. 2011). Spence et al. used a temporal series of growth factor exposure to direct iPSCs from definitive endoderm to hindgut specification/morphogenesis and generate human intestinal organoids (HIOs) in a 3D Matrigel® prointestinal culture system (Spence et al. 2011). The HIOs showed proliferative crypt-like structures projecting from a central cavity with functional

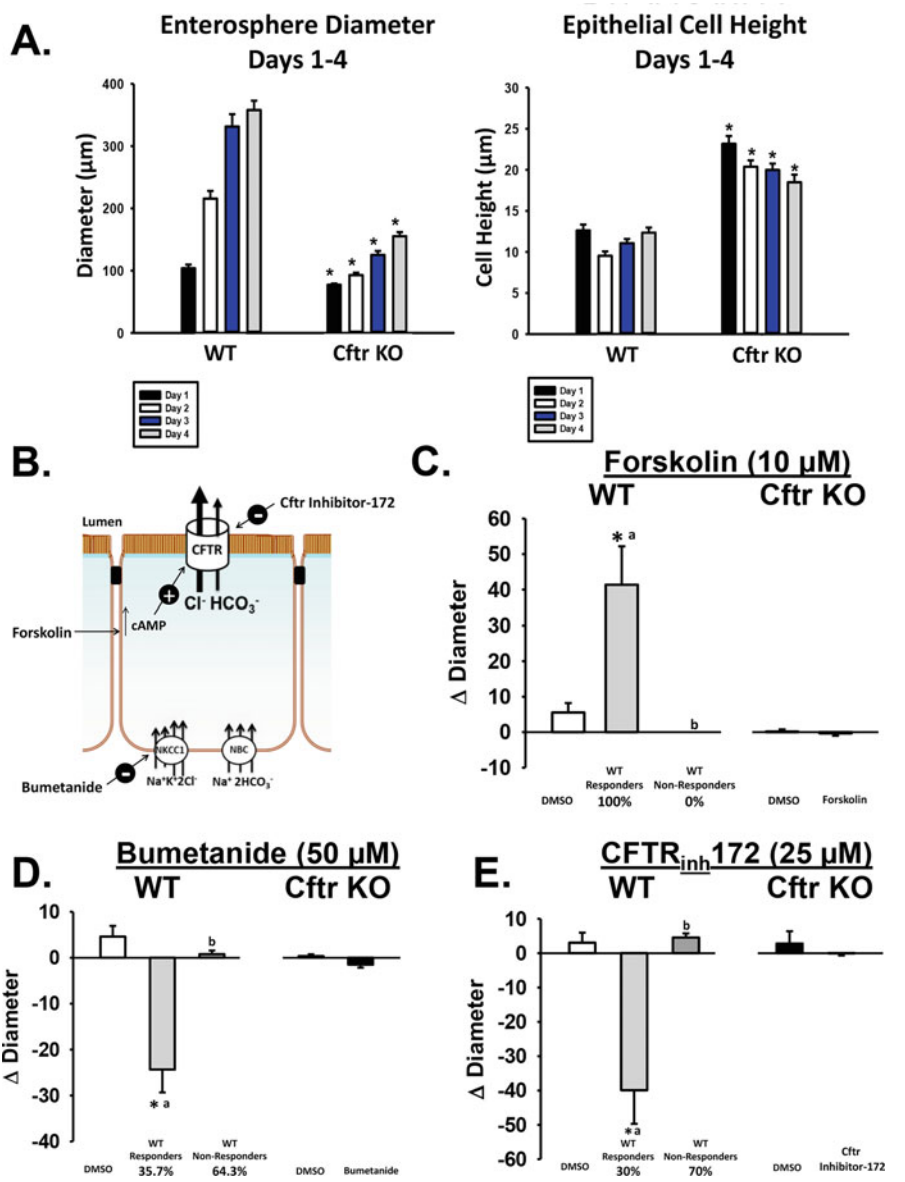

Fig. 1.6 Anion transport in murine enterospheres. (**a**) Time course of WT and Cftr KO enterospheres in culture showing enterosphere diameter and epithelial cell height. *$P < 0.001$ vs. WT, $n = 35$–42 enterospheres from 3 WT/Cftr KO sex-matched littermates. (**b**) Schematic representation of the experiments on Day 2 enterospheres shown in **c**–**e**. (**c**) Effect of forskolin treatment (10 µM, 15 min) on the change in diameter of basal WT and Cftr KO enterospheres. *$P < 0.05$ vs. forskolin-treated Cftr KO. [a,b]Means with different letters are significantly different vs. DMSO control, $P < 0.05$, $n = 8$–11 enterospheres from 3 WT/Cftr KO sex-matched littermates. (**d**) Effect of bumetanide (50 µM, 1 h) on change in diameter of basal WT and Cftr KO enterospheres. Only 35.7% of WT enterospheres responded to bumetanide treatment. *$P < 0.05$ vs. bumetanide-treated Cftr KO. [a,b]Means with different letters are

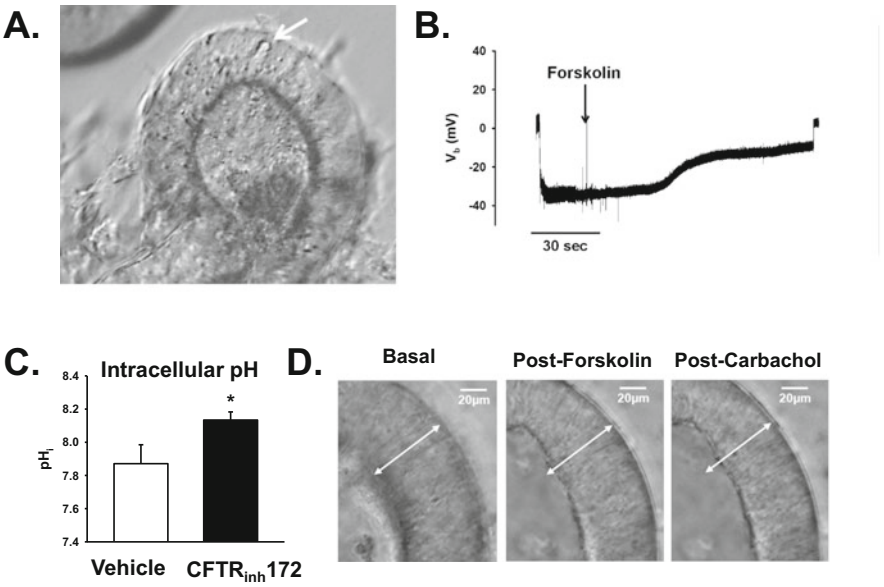

Fig. 1.7 Evidence of CFTR function in intestinal-differentiated iPSCs (HIOs). (**a**) Human intestinal organoid (HIO) from a non-CF, gut-differentiated iPSC (arrow, Paneth cell at crypt base). HIOs were a gift from Dr. James Wells, University of Cincinnati, Cincinnati Children's Medical Center. (**b**) Recording of a microelectrode impalement across the basolateral membrane of a non-CF HIO crypt epithelial cell showing depolarization of the basal membrane potential (V_b) after forskolin (10 μM) addition to the bath, representative of three experiments. (**c**) Effect of CFTR inhibition with CFTR$_{inh}$172 (10 μM, 24 h) on intracellular pH of non-CF HIO crypt epithelial cells. *Significantly different vs. vehicle control (Veh), $n = 4$ different passages. (**d**) Changes in HIO crypt epithelial volume as indexed by changes in cell height following sequential treatments with forskolin (10 μM, 15 min and carbachol (100 μM, 10 min). Bidirectional arrow indicates cell height under basal conditions in all images. Representative of three separate experiments

enterocytes, goblet cells, Paneth, and enteroendocrine cells (Fig. 1.7a). Enterocyte uptake of a fluorescently labelled dipeptide indicated an intact peptide transport system. To investigate CFTR function in the gut-iPSCs, a similar series of experiments were employed as in the murine enteroid study by (Liu et al. 2012). As shown in Fig. 1.7b, microelectrode impalements of non-CF HIO crypt epithelial cells exhibit membrane depolarization after activation of CFTR with forskolin. In

Fig. 1.6 (continued) significantly different vs. DMSO control, $P < 0.05$, $n = 6$–18 enterospheres from 3 WT/Cftr KO sex-matched littermates. (**e**) Effect of CFTR$_{inh}$172 (25 μM, 1 h) on change in diameter of basal WT and Cftr KO enterospheres. Only 30.0% of WT enterospheres responded to CFTR$_{inh}$172 treatment. *$P < 0.05$ vs. inhibitor-treated Cftr KO. [a,b]Means with different letters are significantly different vs. DMSO control, $P < 0.05$, $n = 6$–14 enterospheres from 4 WT/Cftr KO sex-matched littermates

Fig. 1.7c, the regulation of pH_i by CFTR function is assessed by treating the non-CF HIOs with $CFTR_{inh}$-172 for 24 h, which induced cellular alkalization. Figure 1.7d shows the effects of stimulating cAMP and Ca^{2+} mobilization on cell volume regulation in non-CF HIOs, as indexed by lumen expansion and epithelial cell shrinkage. Thus, like murine enteroids, a functional CFTR is present in iPSC-derived HIOs from healthy subjects.

Paralleling the development of HIOs were successful efforts to culture primary human enteroids. Sato et al. found that the method developed to culture mouse enteroids required additional medium supplementation for propagation and prolongation of culture. These supplements included gastrin, nicotinamide, and inhibitors of TGFβ-activin receptors ALK4/5/7and p38 (Sato et al. 2011a). Using similar culture methods, Dekkers et al. explored the ion transport properties of primary human organoids generated from intestinal biopsies of healthy and CF patients. Healthy human enteroids and colonoids responded to forskolin stimulation with organoid swelling, but organoids from CF patients did not (Dekkers et al. 2013). A high-throughput format was developed for CFTR modulator drug screening using intracellular uptake of green fluorescent calcein to accentuate the cellular borders and 2-dimensional imaging (*x*–*y*) to measure changes in organoid area, all within a single culture well. The method was termed the forskolin-induced swelling assay (FIS). This study set the stage for personalized medicine for CF patients by showing that the response of primary human intestinal organoids to FIS in vitro correlated well with ex vivo intestinal short-circuit current measurements in Ussing chambers. Both assays used intestinal biopsies from the same human subjects. In the same year (2013), Donowitz et al. demonstrated that the intestinal organoids from human patient biopsies also exhibited Na^+/H^+ exchanger 3 (NHE3) activity by measuring changes in intracellular pH_i. Treatment of enteroids with the specific NHE3 inhibitor S3226 prevented increased pH_i after an NH_4Cl prepulse used to acidify the cytosol. Because it is known that intracellular cAMP stimulation inhibits NHE3 activity (Donowitz and Welsh 1987), this outcome provided evidence that FIS assay of human enteroids may include inhibition of coupled NaCl absorption (Na^+/H^+, Cl^-/HCO_3^- exchange) in addition to CFTR-mediated anion secretion.

A comprehensive evaluation of ion transport physiology in 3D human intestinal organoids came from studies of upper small intestinal enteroids by Donowitz and others (Foulke-Abel et al. 2016). Studies focused on ion transport and markers of differentiation using Wnt3a stimulation to yield undifferentiated enteroids as a model for crypt-like epithelium and Wnt3a removal to yield differentiated enteroids as a model for villus-like epithelium. In the villus-like epithelial organoids, markers of stem cells (LGR5/achaete-scute complex homolog 2/olfactomedin 4) as well as proliferation indices were decreased relative to the undifferentiated crypt-like enteroids. As expected, markers of lineage commitment such as sucrose-isomaltase and trefoil factor 3 were increased in the villus-like enteroids. In this study, an evaluation of the Na^+/H^+ exchanger NHE3 by measurement of pH_i under conditions that isolate its activity demonstrated that this transporter plays a dominant role in Na^+/H^+ exchange of the enteroid epithelium. Interestingly, NHE3 activity was equivalent between the undifferentiated and differentiated organoids.

Foulke-Abel et al. also used the FIS assay to compare apical fluid secretion between the differentiated and undifferentiated human enteroids. Counterintuitively, the differentiated enteroids showed a large average FIS as compared to the undifferentiated enteroids. As pointed out by the authors, this comparison is confounded by the possibility that paracellular pore pathway in undifferentiated, crypt-like enteroids is leakier than in the differentiated, villus-like enteroids. This conjecture is supported by previous in vivo studies of rat intestine where it was shown that villous pores (<6 Å) of the paracellular pathway are an order of magnitude smaller than crypt epithelial pores (50–60 Å) (Fihn et al. 2000). In a comparison within groups, pretreatment with a combination of pharmacological inhibitors of CFTR ($CFTR_{inh}$-172, GlyH-101) indicated that CFTR is responsible for greater than 90% of FIS in both undifferentiated and differentiated enteroids. Interestingly, pretreatment before forskolin with pharmacological inhibitors of NHE3 (EIPA or S3226) caused a ~30% reduction of the FIS in both enteroid groups. CFTR activation and simultaneous NHE3 inhibition by increases in intracellular cAMP in intestinal epithelium is well documented (Gawenis et al. 2003; Kurashima et al. 1997; Zhao et al. 1999; Yun et al. 1997; Zizak et al. 1999). However, cAMP inhibition of ongoing NaCl absorption as a contribution to FIS is difficult to reconcile with almost complete prevention of FIS by CFTR inhibition. Beyond the possibility of off-target effects of CFTR or NHE3 inhibitors, previous studies have shown that NHE3 inhibition induces the partial activation of anion secretion (likely mediated by CFTR) in rat intestine (Furukawa et al. 2004). It is also known that an acidic pH_i can increase CFTR open probability by increasing the channel's affinity for MgATP (Chen et al. 2009). In the Foulke-Abel et al. study, the authors indicated that acute NHE3 inhibition did not affect enteroid diameter before forskolin, suggesting that inhibition of NaCl absorption may be offset by a suboptimal activation of CFTR. If so, the FIS magnitude during NHE3 inhibition may be reduced by pre-existing CFTR activation, a hypothesis that will require further investigation.

In the same study, bumetanide inhibition of Cl^- uptake by NKCC1 in the FIS assay reduced fluid secretion by 50% in the undifferentiated enteroids and 76% in the differentiated enteroids, indicating a dominant role for CFTR-mediated Cl^- secretion. The fact that inhibition by bumetanide of Cl^- secretion in the FIS assay was incomplete, led Foulke-Abel et al. to speculate that there may be additional Cl^- or HCO_3^- uptake mechanisms involved in FIS. To investigate this aspect, studies of pH_i in differentiated human enteroids were employed to show that cAMP activation of CFTR in the absence of HCO_3^- caused intracellular acidification of pH_i, which was not dependent on NHE3 activity. Forskolin-induced acidification of pH_i was abolished in the presence of HCO_3^-, but was recovered by inhibition of HCO_3^- uptake by the $NaHCO_3$ cotransporter NBCe1. These findings suggest that net HCO_3^- secretion via CFTR activity contributes to FIS of human enteroids, leading to the speculation that net HCO_3^- secretion in cases of secretory diarrhea (e.g., cholera, colibacillosis) may exacerbate systemic dehydration and acidosis.

In a more recent study (Yin et al. 2018), the Donowitz laboratory found that altering the culture conditions affected the ion transport physiology of the 3D human

intestinal organoids in a way more consistent with intact intestine. Although the focus of this report was ion transport in 2D human intestinal enteroid monolayers, studies of transporter expression and FIS in 3D human enteroids were included. The principal change in culture conditions was removal of nicotinamide from the enteroid expansion medium. Although nicotinamide, the amide of the vitamin niacin, is often added to organoid culture to enhance stem cell proliferation, it also has additional activity as an inhibitor of Rho-associated protein kinase (ROCK), by a pathway different than standard ROCK inhibitory Y27632, and as an inhibitor of casein kinase 1 (CK1). The Yin et al. study found, in contrast to the previous Foulke-Abel et al. investigation, that the undifferentiated (crypt-like) 3D human enteroids have an ~two-fold greater FIS as compared to the differentiated (villus-like) enteroids. Moreover, expression profiling of ion transporters indicated that the undifferentiated enteroids had increased expression of CFTR, NKCC1, the cAMP-activated K^+ channel KCNE3 and decreased expression of SLC26A3, NBCe1, the electroneutral NaHCO3 cotransporter NBCn1, carbonic anhydrase 2, and carbonic anhydrase 4, as compared to the differentiated enteroids. With the notable exception of downregulation of NKCC1 in differentiated human enteroids, expression changes for CFTR, SLC26A3, and NBCe1 were not found between undifferentiated and differentiated human enteroids in the Foulke-Abel et al. (2016) study. Although these inconsistencies accentuate the shortcomings of enteroid culture, both studies showed that CFTR-mediated anion secretion and NaCl absorption were present to varying degrees in undifferentiated and differentiated human enteroids. This provides a new perspective on the longstanding controversy regarding "absorptive and secretory compartmentalization" in the small intestine. Some studies contend that an anatomical separation exists between net anion secretion by crypt epithelium and net NaCl absorption by villous epithelium (Hallbäck et al. 1982; Eklund et al. 1987). Other evidence supports the notion that these ion transport processes exist in both (Stewart and Turnberg 1989; De Jonge 1975; Kockerling and Fromm 1993; Singh et al. 1995; Ainsworth et al. 1996; Gawenis et al. 2003). The studies by the Donowitz group suggest the latter is true in isolated, regenerating intestinal epithelium. Although there are differences along the crypt-villus axis in the expression of transporters involved in these processes, it is apparent intestinal epithelial cells have the capacity for both anion secretion and NaCl absorption. The concept of "compartmentalization" of these two important transport processes may be a consequence of differences in activity along a crypt-villus continuum that is accentuated by differences in the epithelial cell number in crypts versus villi. Furthermore, the recent recognition that the small intestine has a subset of "CFTR high expressor" cells, including the villous epithelium, suggests that the concept of compartmentalization may be redefined at a cellular level (Jakab et al. 2013; see Sect. 1.12).

1.4 Precision Medicine of CF Using 3D Human Intestinal Organoid Transport Physiology

The earlier studies by Dekkers et al. (2013) led to the application of organoid ion transport physiology to screen compounds that modulate the activity and processing of mutant CFTR in human rectal organoids from CF patients (Dekkers et al. 2016). The ability to expand the organoids with multiple passages from a single rectal biopsy enabled the testing of different combinations and dosages of recently developed pharmaceutical modulators of CFTR, thus ushering in personalized medicine for CF patients. Over 2000 *CFTR* mutations have been identified worldwide (www.genet.sickkids.on.ca) with variances in phenotype, including single-organ CFTR-deficient diseases such as some cases of idiopathic chronic pancreatitis (Noone et al. 2001). Driven by the difficulties of testing pharmacologic therapy in CF patients with so many different mutations, especially those with rare mutations, these investigators used rectal organoids to test two CFTR modulators initially developed for a limited number of CFTR mutations. The first modulator registered for treating CF patients carrying *CFTR* mutations with defective channel gating (G551D, S1251N, others) was the CFTR potentiator ivacaftor (Kalydeco®, Vertex Pharmaceuticals) (Ramsey et al. 2011; Accurso et al. 2010; De Boeck et al. 2014). This was shortly followed by the introduction of the protein processing corrector lumacaftor, which in combination with ivacaftor, is known as ORKAMBI ® (Vertex Pharmaceuticals). The latter was shown in clinical trials to have beneficial effects on lung function in CF patients with the most common CF disease causing mutation, ΔF508 (Boyle et al. 2014; Wainwright et al. 2015).

The study employed the FIS assay as described here but found that the organoid phenotype (enlarged cysts) from biopsies of healthy patients or CF patients with mutations showing high residual function negatively correlated with the FIS response. To address this difficulty, Dekkers et al. (2016) introduced the steady-state lumen area assay (SLA), which basically involves confocal microscopic measurement of the total organoid area and the luminal area in the *x*–*y* plane in organoids under basal, steady-state conditions (without forskolin treatment). The SLA is expressed as the luminal organoid area as a percentage of the total organoid area. SLA could readily discriminate organoids from healthy subjects with at least one WT CFTR allele (SLA $\geq$ 50%) and organoids from CF patients with class I–V CFTR mutations (SLA 0–10%). Among CF organoids, the SLA assay could also discriminate between class I–III CFTR mutations (no CFTR synthesis, defective trafficking, and defective channel gating, respectively) with SLA = ~0–0.1% and class IV–V CFTR mutations (altered channel conductance or reduced amounts of CFTR protein) with SLA = ~5%. It was shown that large SLA organoids (>40%) from healthy subjects with at least one WT CFTR allele was negatively correlated with the increase in organoid area in the FIS. In contrast, the FIS assay, which extends over 60 min, was well correlated in organoids with lower SLA (<40%). This indicated that the FIS likely underestimates WT CFTR response to forskolin, thereby obviating a direct comparison to CF organoids. However, the SLA of all CF

organoids was below 10%, therefore supporting the utility of the SLA assay for testing CFTR modulator therapy.

The poor response to FIS in Wnt-treated, non-CF rectal organoids (SLA >40%) in the Dekkers et al. (2016) studies has some parallels with observations of Wnt3a-treated WT murine enterospheres (see Fig. 1.6). These studies suggest that CFTR is active at some point during Wnt-stimulated growth and perhaps inhibited upon spheroid maturation. As shown in Fig. 1.6a, the rapid increase in enterosphere diameter during Days 2–3 in culture is nearing a plateau by Days 3–4. Indeed, the FIS assay of murine enterospheres was performed on culture Day 2 because older, enlarged WT enterospheres had diminished FIS responses similar to the findings with the human rectal organoids (data not shown). CFTR activity during stimulated growth is largely responsible for organoid swelling, but its activation negatively regulates and slows proliferation (Strubberg et al. 2018; Than et al. 2016). As mentioned previously, the nonresponders to Cftr inhibition (70%, Fig. 1.6e) in Day 2 WT enterospheres suggest that proliferation regulation may change with time in culture, likely in response to several factors during Wnt-stimulated organoid growth. For example, rapid growth of dividing cells (ISCs, transit-amplifying cells) may activate CFTR function to slow proliferation, but the consequent organoid swelling may exert negative influences on CFTR activity. Given the squamous morphology of cells constituting a mature, enlarged spheroid, the lack of response to forskolin may include alterations in CFTR or other transporters as a consequence of backpressure and cell stretching (Vitzthum et al. 2015). Further research in this area is needed because it is important to understand the underlying ion transport physiology of Wnt-stimulated organoids because of their utility for drug screening and, in the case of enterospheres, as an index of ISC function. However, it should be borne in mind that these studies involved *acute* pharmacological manipulation of organoids. Longer-term studies using SLA or FIS may be more difficult. Videomicroscopic recordings of enterospheres over a 24-h. period found that distended WT enterospheres undergo several episodes in which the epithelium ruptures, the organoid reduces in volume, the epithelium reseals, and the organoid swells again. Thus, the quantitative aspect of swelling assays may be confounded in longitudinal drug studies.

1.5 Advantages of Studies of Ion Transport in 3D Intestinal Organoids: A Summary

1. Organotypic morphology: One of the most exciting features of enteroid culture is the organotypic features of a central cavity from which crypts project, i.e., "mini-gut." At the cell level is the formation of a polarized, well-differentiated intestinal epithelium with approximately the same percentage of cells of different lineages as found in vivo (Sato et al. 2009). Unlike cell lines, 3D organoids provide the opportunity to study the electrophysiology and ion transport capabilities of

intestinal epithelium in situ, in particular, the crypts. The verisimilar nature of the 3D organoids is highlighted by the capability for functional, long-term engraftment of intestinal organoids to sites of damaged colonic epithelium in a mouse model (Yui et al. 2012). This raises the possibility for autologous intestinal engraftment by in vivo differentiated HIOs produced from nondiseased tissues of a patient.

2. Visual access: A remarkable aspect of 3D intestinal cultures is the visual access to crypt function. The opportunity to view, by either light or fluorescence microscopy, the dynamics of a regenerating crypt epithelium in axial or coronal planes advances the ability to investigate ion transport properties in real time. As mentioned previously, it enables studies of ISCs for intracellular pH and ion concentrations using fluorescent dyes, provides electrophysiological access for microelectrode impalements or patch clamping of the basolateral membrane, and allows direct visualization of crypt and ISC cell volume regulation. Studies of ion transport physiology of other differentiated cell types are possible as shown by measurements of goblet cell pH_i, mucin granule pH, and the dynamics of degranulation in situ (Liu et al. 2015).
3. Isolation from the intestinal milieu: Intestinal organoids are growing epithelial structures removed for weeks or even months (in passage) from the intestinal environment. The advantage is that removal from neural, immune, humoral, and microbial influences enables the investigation of ion transport processes that are intrinsic to the epithelium and not secondary to other effects of disease, such as dysbiosis. We have found that some indicators of inflammation in the CF mouse intestine, e.g., Toll-Like receptors, and goblet cell hyperplasia are normalized by the first passage in enteroid culture (unpublished observations). This aspect alone raises the opportunity to assess epigenetic changes in ion transport physiology in disease states by following changes with time in culture.

1.6 Disadvantages for Studies of Ion Transport in 3D Intestinal Organoids

1. Access to the brush-border membrane: Given the vectorial nature of ion transport processes in a polarized epithelium, access to the brush-border membrane in intact organoids presents limitations to the efficiency and types of experiments. The containment of the brush-border membrane within the organoid prevents direct contact with micropipettes without disrupting the epithelial structure for access. Compounding this limitation is the presence of cell debris and even mucus (depending on the treatment protocol) within the organoid's lumen. Intestinal organoids are elastic and distensible, so it is possible to inject (Engevik et al. 2013) and even superfuse (Liu et al. 2015) the organoid lumen with compounds, isotopes, or different solutions to enable investigation of ion transport processes. However, these studies are time consuming and technically demanding. Bisection of organoids provides a means to allow access to the apical membrane for ion

transport probes or solutions, but with the limitation that both apical and basolateral membranes of the epithelium are exposed to the same milieu (Liu et al. 2015).

2. Limitations of the gel: The virtues of 3D culture of intestinal organoids within a gel (e.g., Matrigel®) include a laminin basement membrane that avoids anoikis and a gel structure enabling cell migration to self-organize into a 3D organoid. However, the gel provides limitations with regard to diffusibility of compounds or reagents, especially those of a hydrophobic nature. This limitation leads to heuristic efforts for finding optimal responses usually of an empirical nature such as dose-response or time-course studies for comparisons with published values from native tissue or cell line experiments. The same is also true about the washout of compounds/reagents from the gel in these studies. Though practical solutions are possible, the additional time required to develop an experiment presents an inefficiency. With regard to some classic approaches to ion transport studies, a qualitative rather than a quantitative outcome might also be a consequence of an experiment with 3D organoids. For example, a study of the basolateral Cl^-/HCO_3^- exchanger Ae2 in mouse intestinal organoids found an exchange rate that was lower than that established from studies of cell lines. The inconsistency was ascribed to the slow turnover of solutions within the gel during switches from Cl^--containing to Cl^--free solutions employed to drive the exchange process (Walker et al. 2016). In addition to the problem of drug/compound access to the organoid and their washout, the structure of the gel itself can be problematic in some treatments. For example, in the aforementioned study, the duration of the experiment was limited because the Matrigel® tended to dissolve and loosen in the culture dish during superfusion with the Cl^--free solution. Likewise, our laboratory has found that solutions with high K^+ concentrations to have a similar destabilizing effect on the gel.

Together, these limitations to ion transport studies in the 3D gel cultures of intestinal organoids have driven efforts to establish 2D monolayers of primary intestinal epithelium, i.e., 2D organoids.

1.7 Ion Transport in 2D Intestinal Organoids: Converting 3D into 2D Intestinal Organoids

Following their amplification as spheroids by multiple passaging, stem cell–derived 3D enteroids and colonoids from animal (mouse, pig) or human origin embedded in Matrigel droplets can be readily converted into confluent monolayers of polarized epithelial cells. Removal of the Matrigel is followed by trypsinization, mechanical disruption, straining through a 40-μm filter, and plating of the near-single cell fragments in expansion medium on a semipermeable substrate (Transwell or Snapwell filter inserts, Corning). Precoating of the inserts has been performed with a fibronectin-based peptide (Foulke-Abel et al. 2014), human collagen IV (In et al.

2016; Yin et al. 2018; Fernando et al. 2017; van der Hee et al. 2018), 1–2.5 vol% Matrigel (Wang et al. 2019; VanDussen et al. 2015), PureCol (Advanced Biomatrix) (Zomer-van Ommen et al. 2018), or Matrigel covered by a feeder layer of irradiated 3T3 fibroblasts (Wilke et al. 2019). In most protocols, expansion medium is supplemented with the ROCK inhibitor Y-27632 (to prevent anoikis) and the GSK3 inhibitor CHIR99021 (to promote Wnt/β-catenin signaling) during the first 2 days after seeding. Once monolayers become confluent, as judged by their morphology and transepithelial electrical resistance (TEER) requiring 7–14 days, they can be maintained in expansion medium for another 4–6 days, resulting in stem cell-enriched, undifferentiated 2D organoid monolayers. Of note, the cell composition of these undifferentiated monolayers differs from the native intestinal crypts or 3D enteroids in that secretory cell types, including mucus-secreting goblet cells and enteroendocrine cells (EEC), are underrepresented or completely missing.

However, 2D intestinal organoids can be enriched for absorptive and secretory cell types by growing them in differentiation medium made by withdrawing niche factors, in particular Wnt3a, the p38 MAPK inhibitor SB202190, and, occasionally, nicotinamide or R-spondin1 from the expansion medium (Yin et al. 2018; Zomer-van Ommen et al. 2018), or by replacing Wnt3a by the Wnt pathway inhibitor IWP-2 (Yin et al. 2014). During differentiation, the TEER value increases from ~200 to ~1400 Ohm.cm^2, i.e., appr. seven-fold (In et al. 2016). A further enrichment for goblet cells can be achieved by adding the Notch inhibitor DAPT (Yin et al. 2014) or by growing the monolayer under air-liquid interface (ALI) conditions, creating a thick mucus hydrogel layer. As shown in several recent studies, this condition offered a suitable platform for the efficient growth of *E. coli* microbes and *Cryptosporidium* parasites (Wang et al. 2019; Wilke et al. 2019; Heo et al. 2018). Alternatively, differentiation of stem cells into enteroendocrine cells (EEC), including GLP1-producing L-cells, can be reached by blockage of both the Notch and the EGFR/MAPK signaling pathway (Basak et al. 2017) or by inhibiting RhoA/ROCK/YAP signaling using the ROCK inhibitor Y-27632 (Petersen et al. 2018). It should be noted, however, that the latter studies have been carried out with murine enteroids only and that confirmation of these results for human organoids needs further experimentation.

1.8 Advantages and Limitations of 2D Organoids

Two-dimensional monolayers of enteroids and colonoids, in contrast to 3D organoids [oriented either basal-out or apical-out; cf. Co et al. (2019)], are freely accessible at both the basolateral (serosal) side and the apical (luminal) side, therefore bypassing the need for microinjection of test compounds or microbes. They greatly expand the applicability of intestinal organoids as ex vivo models in studies of epithelial permeability, mucus production and secretion, electrolyte and fluid transport, and interaction of epithelial cells with microbes, parasites, and microbial enterotoxins. Organoid monolayers enriched for specific cell types (e.g.,

goblet cells; EEC; tuft cells) can be used for studying apical vs. basolateral secretion of their content in response to secretory stimuli, including those of pathogens at an early stage of infection. Moreover, unlike transformed human intestinal cell lines (T84, HT29, CaCo2), they allow the study of segmental differences and spatial differences along the crypt-villus axis at both a macroscopic and microscopic level (Middendorp et al. 2014; Dutton et al. 2019).

Another advantage in comparison with most cell lines is the ability of goblet cell–enriched organoid monolayers to build up a mucus layer at their luminal side, which mimics the barrier function of native epithelium (Wang et al. 2019). By comparing CFTR-mediated anion currents across monolayers of undifferentiated, mucus-free organoids with mucus-enriched organoids, the impact of the mucus barrier on the efficacy of luminally added compounds, including CFTR inhibitors (relevant in diarrheal disease) and CFTR activators (relevant for CF), can be assessed. Moreover, approaches that facilitate transport of drugs across the mucus layer, such as alginates or nanoparticles, can be evaluated (Ermund et al. 2017; Davoudi et al. 2018).

Obvious shortcomings of the 2D minigut are the absence of a 3D crypt-villus architecture and consequently its inability to recapitulate in vivo cell migration, cell extrusion [a favorable infection site; cf. Co et al. (2019)], and hormone or growth factor gradients along the crypt-villus axis. Other limitations and pitfalls are the potential entrapment of compounds by the filter; the low throughput of transport assays in comparison with the FIS assay performed in 96-or 384-well plates; the lack of a mesenchyme, myofibroblasts, immune cells, endothelial cells, smooth muscle cells and enteric nerves; and the lack of peristalsis, luminal flow, and blood circulation (In et al. 2016). One approach to compensate for these limitations is to coculture 2D epithelial monolayers with such key cell types and to mimic peristalsis and flow in a microengineered gut-on-a-chip device (Kim and Ingber 2013; Kim et al. 2016, 2017; Bein et al. 2018).

1.9 Ion Transport Studies in Undifferentiated 2D Human Intestinal Organoids

As is apparent from their transcriptome profile and histological analysis, human rectal organoids grown in the presence of Wnt3a and other growth factors, similar to 2D human duodenal enteroids (Yin et al. 2018), are nearly devoid of mucin 2 (MUC2)-secreting goblet cells and express very low levels of the apical Na^+/H^+ exchanger NHE3 and the Cl^-/HCO_3^- exchanger DRA; in contrast, they are highly enriched in the stem cell marker Lgr5 and in ion transporters and channels involved in transepithelial secretion of chloride, i.e., NKCC1 and CFTR (Table 1.1; cf. Fig. 1.6b). Monolayers of colonoids are therefore excellently suited to study electrogenic anion secretion through short-circuit current (I_{sc}) measurements in conventional Ussing chambers or equivalent short-circuit current (I_{eq}) measurements in a 24-well MTECC system (EP-devices) (Zomer-van Ommen et al. 2018). They

Table 1.1 RNA-Seq of ion channels, transporters, and transport regulators expressed in undifferentiated, differentiated, and goblet cell–enriched human colonoids

	Transcript levels in human colonoids (RPKM)		
Gene	Undifferentiated	Differentiated	Goblet cell–enriched
LGR5	10.6	0.3	0.2
MUC2	4.4	44.2	136.1
CFTR	15.7	1.2	3.1
NKCC1/SLC12A2	125.2	8.8	14.6
NBC1/SLC4A4	1.6	3.9	3.4
NHE3/SLC9A3	2.1	8.2	10.1
DRA/SLC26A3	2.1	8.2	10.1
ANO1	0.2	ND	0.1
BEST2	ND	ND	ND
WNK1	16.2	5.5	6.7

Colonoids generated from rectal suction biopsies of healthy volunteers and grown in Matrigel droplets were cultured for 7–9 days in WENR medium (containing Wnt3a, EGF, Noggin, and R-spondin1, promoting the undifferentiated state), ENR medium (containing the WNT inhibitor IWP-2, resulting in differentiation), or ENR + IWP-2 + DAPT medium [promoting goblet cell-enrichment (Yin et al. 2014)]. RNA extraction and transcriptome sequencing was performed as described in detail elsewhere (Ikpa et al. 2020). Data depict reads per 1000 base pair transcript per million reads mapped (RPKM). *ND* not detected

can also be used for the screening of novel antidiarrheals, including CFTR inhibitors and guanylyl cyclase C (GUCY2C) inhibitors (Bijvelds et al. 2015). Moreover, 2D colonoids generated from rectal biopsies of CF individuals can be used to investigate the residual function of CFTR mutants and their response to CFTR modulators with more quantitative electrophysiological measurements (Zomer-van Ommen et al. 2018).

In comparison with intestinal short-circuit current measurements (I_{sc}) in freshly excised, near-native human rectal biopsies, 2D colonoids offer several major advantages: (1) their availability, through biobanking and expansion of 3D organoids, in almost unlimited amounts; (2) CFTR current responses to cAMP-linked agonists (e.g., forskolin, IBMX) are two- to threefold larger than in the biopsies of the same individual despite the flat structure of the monolayer, which comprised a much smaller number of cells per cm^2 (Fig. 1.8a vs. b). This difference is due, at least in part, to the enrichment of stem cells in the colonoids, which express high levels of CFTR (Strubberg et al. 2018), and to the finding that CFTR currents in biopsies, in contrast to colonoids, are no longer proportional to the amount of CFTR above ~20–30% of WT levels, as based on Isc and CFTR band C quantification in biopsies of CFTR splice mutants (De Boeck et al. 2014); (3) the near absence of goblet cells and mucus in the undifferentiated colonoids. The absence of a fully formed mucus layer may facilitate uptake of hydrophobic compounds, e.g., CFTR inhibitors and modulators, in cells from the luminal side. This, together with the lack of convectional washout of CFTR-targeted drugs from 3D crypts, may explain why CFTR inhibitors are highly effective in colonoids but poorly effective in rectal biopsies and

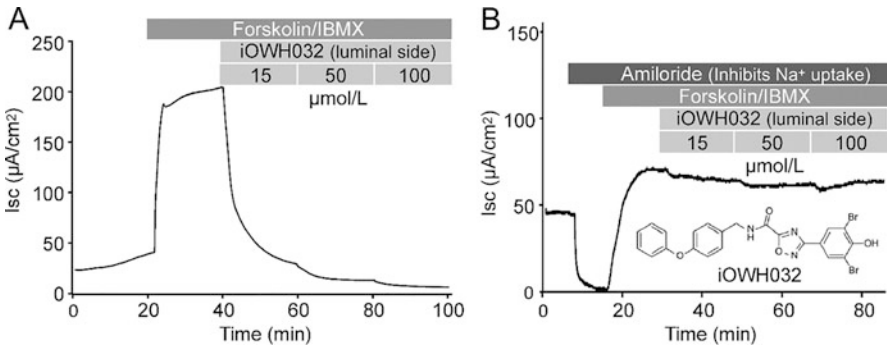

Fig. 1.8 Comparison of CFTR-mediated anion secretory currents in 2D human rectal colonoids (**a**) vs. human rectal biopsies (**b**). (**a**) Undifferentiated colonoid monolayers derived from human rectal biopsies were grown on Transwell filters and subsequently mounted in Ussing chambers. Short-circuit currents (I_{sc}), representing electrogenic ion transport, were assessed as described in detail elsewhere (Bijvelds et al. 2009). (**b**) Rectal biopsy specimens were obtained from healthy volunteers. Biopsies were mounted in Ussing chambers and the I_{sc} was measured as described for panel (**a**). Amiloride (10 µM) and the CFTR inhibitor iOWH032 were added only to the luminal bath; forskolin (10 µM) and IBMX (100 µM) were added to both the luminal and basolateral bath

in intact epithelium [Fig. 1.8a vs. b; cf. Thiagarajah and Verkman (2013)]. Furthermore, the absence of goblet cells in colonoids readily explains why electrogenic K^+ secretion in response to Ca^{2+}- or cAMP-linked secretagogues, a property attributed exclusively to goblet cells in distal colonic crypts (Linley et al. 2014), is very prominent in rectal biopsies of CF individuals but not manifest in 2D colonoids generated from these biopsies (Fig. 1.9a, b). The absence of active K^+-driven fluid secretion also explains why 3D colonoids expressing CFTR-null mutants do not swell in response to cAMP- or Ca^{2+}-linked secretagogues (Dekkers et al. 2016); (4) they can be used for prolonged ex vivo experiments, viz. for testing slow-acting small-molecule compounds that improve the folding of de novo synthesized mutant CFTR. Because optimal restoration of mutant CFTR activity by folding correctors typically requires >8 h incubation, such assays cannot be performed in intestinal biopsies, as these remain fully viable only for a limited period, typically <6 h.

1.10 CFTR Is the Major If Not Sole Apical Anion Channel Accounting for cAMP- and Ca^{2+}-Activated Electrogenic Anion Secretion in 2D Colonoids

In 2D, human colonoids both Cl^- and HCO_3^- secretory currents induced by the cAMP agonist forskolin are almost fully blocked (>90%) by the CFTR inhibitors iOWH-32 and $CFTR_{inh}$-172 (Fig. 1.10a–c). In accordance, these secretory currents are virtually absent from CF colonoids derived from patients with CFTR mutations

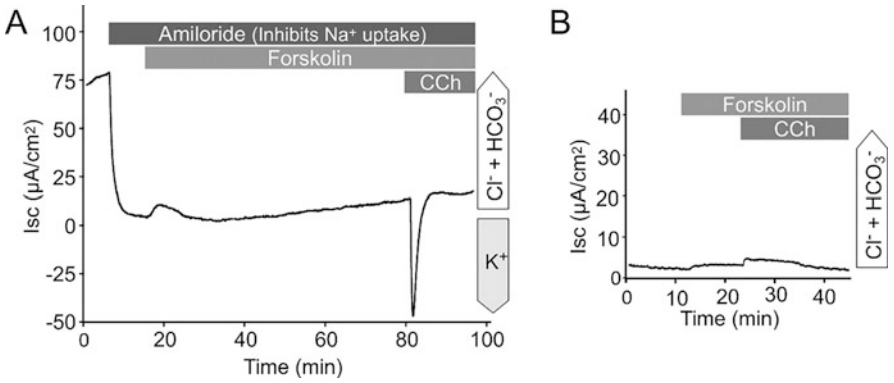

Fig. 1.9 Carbachol-activated K^+ secretion is prominent in CF rectal biopsies but absent in 2D CF colonoids. (**a**) Rectal biopsy specimens, obtained from CF patients (homozygous F508del), were mounted in Ussing chambers. Short-circuit currents (I_{sc}), representing electrogenic ion transport, were assessed as described in detail elsewhere (Bijvelds et al. 2009). (**b**) Undifferentiated colonoid monolayers derived from CF rectal biopsies (homozygous F508del) were grown on Transwell filters and subsequently mounted in Ussing chambers. The Isc was measured as described for panel (**a**). Amiloride (10 μM) was added only to the luminal bath; Carbachol (CCh; 100 μM) was added only to the basolateral bath; forskolin (10 μM) was added to both the luminal and basolateral bath

that severely impair ion transport function and/or protein maturation and membrane trafficking (e.g., homozygous F508del; Fig. 1.9b).

Addition of a Ca^{2+}-mobilizing secretagogue (carbachol; UTP) after CFTR channel blockade did not provoke anion secretion in undifferentiated colonoids (Fig. 1.11a) or enteroids (not shown), and triggered only a very small anion current in differentiated and goblet cell–enriched colonoids, arguing against a substantial contribution of apical Ca^{2+}-activated anion channels (CaCCs) to this process (Fig. 1.11a). In contrast, the application of UTP to 2D human biliary duct organoids clearly demonstrated the contribution of a Ca^{2+}-activated apical anion channel to Cl^- and HCO_3^- secretory currents in this model (Fig. 1.11b). Of note, the high remnant Ca^{2+}-dependent anion secretion previously observed in the colon of Cftr KO mice, thought to reflect bestrophin-2 (Best2) channel-mediated bicarbonate secretion from goblet cells (Yu et al. 2010), is not found in goblet cell–enriched human colonoids, in line with the virtual absence of *BEST2* transcripts (Table 1.1). Similar studies in mouse colonoids may reveal whether this finding reflects an interspecies difference or a limitation of the organoid model.

The outcome of these experiments on 2D human enteroids and colonoids is in line with a recent study showing that, whereas the CaCC TMEM16A/ANO1 is expressed in the mouse intestine, it is not directly involved in transepithelial anion secretion or required for mucus homeostasis (Vega et al. 2019). This study also suggests that the large contribution of CaCCs to Ca^{2+}-induced anion secretion reported previously for human colorectal tumor cells (T84; HT-29cl.19A) results from an upregulation of CaCCs during intestinal tumorigenesis (Bajnath et al. 1992; Namkung et al. 2011;

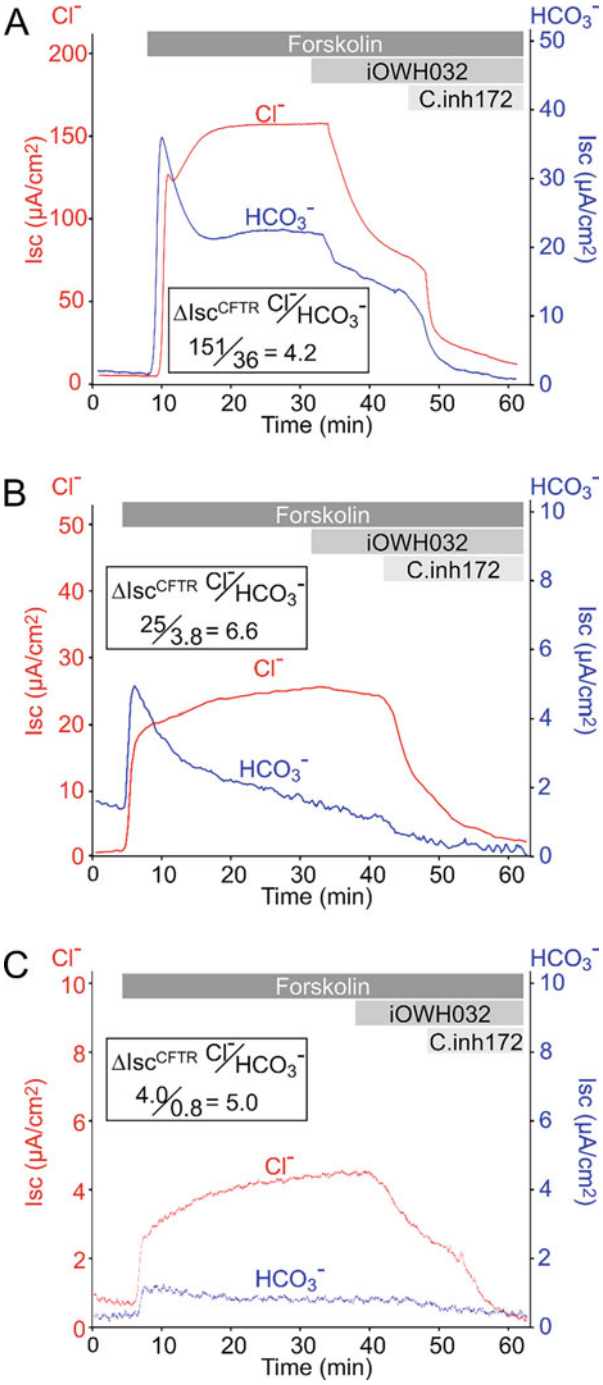

Fig. 1.10 CFTR-mediated Cl^- and HCO_3^- current responses to forskolin/cAMP in 2D human rectal organoids at various differentiation stages. (**a**) Undifferentiated; (**b**) Differentiated; (**c**) Goblet cell enriched, all derived from a healthy donor. Electrogenic Cl^- secretion was measured in a HEPES-buffered, HCO_3^--free (replaced by isethionate) isotonic solution. HCO_3^- secretion was measured in a Cl^--free (replaced by isethionate) isotonic solution. $CFTR_{inh}$-172 (C.inh172; 20 μM) and iOWH032 (30 μM) were added only to the luminal bath; forskolin (10 μM) was added to both the luminal and basolateral bath

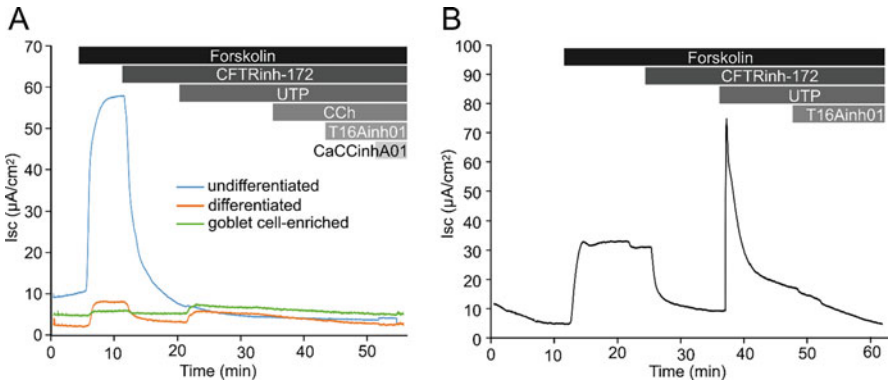

Fig. 1.11 Regulation of anion secretion in 2D cultured organoids. (**a**) Anion secretory currents mediated by cAMP- (forskolin) and Ca^{2+}- (carbachol, UTP) agonists in 2D human undifferentiated, differentiated, or goblet cell–enriched colonoids. (**b**) Anion secretory currents in human bile duct organoids. UTP (50 µM), $CFTR_{inh}$-172 (20 µM), TMEM16A inhibiter A01 ($T16A_{inh}01$; 25 µM), and CaCC inhibitor A01($CaCC_{inh}A01$, 25 µM) were added to the luminal bath only; forskolin (10 µM) was added to both the luminal and basolateral bath; carbachol (CCh; 100 µM) was added to the basolateral bath only

Sui et al. 2014). This view conflicts with other recent publications, arguing that, although ANO1 does not function as an intestinal apical chloride channel, it nevertheless is required for cAMP-dependent colonic chloride and fluid secretion, and that ANO1 blockers or activators can potentially be used to treat enterotoxin-related diarrhea or CF-related intestinal disease, respectively (Benedetto et al. 2017; Lee et al. 2019; Kunzelmann et al. 2019). Functional experiments in human intestinal organoids, using CRISPR-Cas9-based silencing of the ANO1 gene, may help to resolve this controversy. So far, the results of the organoid studies support the earlier notion that Ca^{2+} agonists act mainly, if not exclusively by activating the basolateral K^+ channel SK4 (Matos et al. 2007; Flores et al. 2007). The resulting membrane hyperpolarization enhances the electrical driving force for anion exit through apical CFTR channels (cf. Fig. 1.12; Greger 2000). Therefore, in contrast to bile ducts, human intestine cannot compensate functionally for defective CFTR channels by exploiting alternative channels, in line with the early onset and severity of CF intestinal disease (e.g., meconium ileus).

1.11 2D Organoids Allow the Separate Measurements of CFTR-Mediated Cl^- and HCO_3^- Secretion in Colonocytes at Different Stages of Maturation

To mimic physiological conditions, in short-circuit current measurements of intestinal biopsies or 2D organoids, the mucosal and serosal baths contain Cl^- and HCO_3^-, which both contribute to the anion secretory current. A simple approach

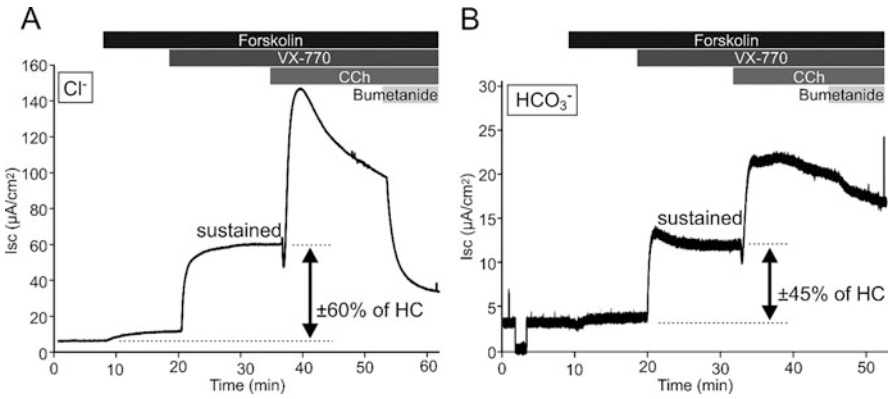

Fig. 1.12 VX-770 rescue of anion secretion in 2D human CF colonoids. Rescue of CFTR-mediated, forskolin (10 μM)-dependent Cl^- (panel A) and HCO_3^- (panel B) secretion in 2D human CF colonoids carrying the S1251N gating mutation by VX-770 (3 μM). The Ca^{2+} agonist carbachol (CCh; 100 μM) further stimulated the CFTR-mediated current. Bumetanide (50 μM) inhibited the Cl^--, but not the HCO_3^--current. The regain of the forskolin-activated Cl^- and HCO_3^- current in the S1251N organoids reached a value of 60% and 45%, respectively, of the current measured in non-CF organoids (HC) in the absence of CFTR modulators. See legend Fig. 1.10 for composition of bath solutions

to separate both currents is to perform I_{sc} measurement using a Cl^-- or HCO_3^- -free bath composition (Yin et al. 2018). Furthermore, the depletion of Cl^- in the luminal bath prevents the operation of the apical Cl^-/HCO_3^- exchanger SLC26A3 and thereby facilitates HCO_3^- exit through the CFTR channel. To perform this approach in 3D organoids is more cumbersome, because in this model, the ion composition of the luminal fluid is difficult to manipulate experimentally.

As shown in Fig. 1.10a–c, both the forskolin-activated, CFTR-mediated Cl^- current and HCO_3^- current decreased dramatically during differentiation and even further following DAPT treatment, in parallel with the drop in CFTR gene expression (Table 1.1). Remarkably, differentiation did not notably change the HCO_3^-/Cl^- current ratio (~0.2). This finding supports the notion that, in 2D colonoids, CFTR rather than basolateral Cl^- and HCO_3^- import through NKCC1 and NBCe1, respectively, is rate limiting for the current (cf. Table 1.1). Previous studies on ductal cells from the pancreas suggest that the permselectivity of CFTR for Cl^- and HCO_3^- ions is not invariable but dynamically regulated by WNK1, a protein kinase capable of binding to the N-terminus of CFTR at low intracellular Cl^- and shifting the HCO_3^-/Cl^- permeability ratio of the channel ~fivefold, i.e., from ~0.2 to 1.0 (Kim et al. 2020). Assuming that this mode of regulation is also operating in cultured intestinal colonoids (in which WNK1 is highly expressed; see Table 1.1), it appears that WNK1 activation does not notably enhance HCO_3^- secretion in this model. We speculate that the HCO_3^-/Cl^- current ratio measured under these conditions principally reflects a difference in the electrochemical driving force for extrusion of these ions across the apical membrane (i.e., $Cl^- >> HCO_3^-$). However,

after selective permeabilization of the basolateral membrane by pore-forming peptides (e.g. nystatin), apical ion gradients can be experimentally manipulated. Under such conditions, the application of Cl^- or HCO_3^- concentration gradients across the monolayer allows for direct assessment of the HCO_3^-/Cl^- permeability ratio (Bijvelds et al. 2009). Such macroscopic current measurements may supplant measurements of the HCO_3^-/Cl^- permeability ratio of CFTR at a single cell level by the whole-cell patch-clamp technique and may reveal whether CFTR mutations or CFTR modulators, by affecting the conformation or accessibility of the channel pore, not only change the activity of CFTR but also modulate its permselectivity for anions (Ferrera et al. 2019).

1.12 Contribution of CFTR-High Expressor Cells to Ion Transport in Intestinal Organoids

A small subpopulation (~2.5%) of enterocyte-like epithelial cells that express extremely high levels of CFTR in their apical membrane named CFTR High Expressor (CHE) cells have been identified in duodenum and jejunum of human and rat, but not mouse intestine (Jakab et al. 2013). These cells resemble so-called pulmonary ionocytes identified more recently by single cell transcriptomics in human and mouse airways that have been claimed to function as a major source of CFTR activity in airway epithelium, despite representing only 1–2% of the epithelial cell population (Plasschaert et al. 2018). In both cases, the function of CFTR has not yet been finally resolved, awaiting new approaches to enrich cell preparations or organoids for these rare cell types. So far however the occurrence of CHE cells in human duodenal or jejunal enteroids has not been reported, hampering the use of organoids as a new and permanent source for studies of this intriguing cell type. It should be pointed out however that the level of CFTR-mediated anion secretory current measured in enteroids generated from the proximal (CHE-containing) and distal (CHE-less) small intestine is very similar, suggesting that CHE cells do not contribute much to transepithelial anion transport in the human intestine but are likely to serve other functions.

1.13 Testing of CFTR Correctors and Potentiators in 2D Colonoids and Enteroids Generated from Intestinal Biopsies of CF Patients

Two-dimensional intestinal organoids are not only suitable as an in vitro model for measuring the impact of CFTR mutations on intestinal Cl^- and HCO_3^- secretion, but, similar to the 3D organoids discussed earlier, can be exploited too, albeit at a much lower throughput, for theratyping CFTR mutants, in particular compound

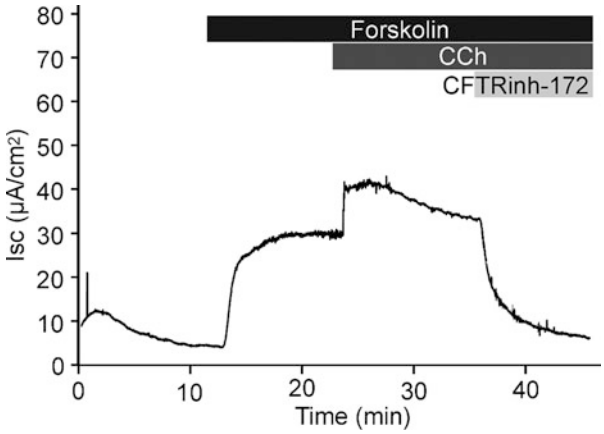

Fig. 1.13 TRIKAFTA rescue of anion secretion in 2D human CF colonoids. Rescue of CFTR-mediated anion secretion in 2D human colonoids (homozygous F508del; undifferentiated) by the triple combination of 2 Vertex correctors (VX-661 + VX-445) *plus* the Vertex potentiator (VX-770), named TRIKAFTA. The colonoid monolayers were preincubated for 20 h in the presence of TRIKAFTA or vehicle (0.1% DMSO). $CFTR_{inh}$-172 (25 μM) was added to the luminal bath only; forskolin (10 μM) was added to both the luminal and basolateral bath; carbachol (CCh; 100 μM) was added to the basolateral bath only

heterozygotes, using existing and candidate CFTR modulator drugs. A typical example is illustrated in Fig. 1.12a, b. It shows that the CFTR gating mutant S1251N can regain up to 60% of the Cl^- and 45% of the HCO_3^- secretory activity of healthy control-derived colonoids following addition of the potentiator VX-770/Ivacaftor (Vertex Pharmaceuticals). As shown in Fig. 1.13, a similar efficacy of CFTR rescue of the most common CFTR mutation F508del, suffering from multiple defects, including impaired protein folding, trafficking, and gating, required a combination of VX-770 with two folding correctors, VX-445/Elexacaftor and VX-661/Tezacaftor, named TRIKAFTA® (Vertex Pharmaceuticals). A high efficacy of CFTR rescue was also observed in 2D ileal organoids from homozygous F508del CF patients (not shown). Both CFTR modulator treatments are FDA approved and have shown to be highly effective in the clinic (Middleton et al. 2019; Heijerman et al. 2019), underscoring the predictive value of the in vitro functional tests in 2D organoids.

1.14 Concluding Remarks

Up until the advent of the organoid technology developed after 2000, intestinal epithelial cells were notoriously difficult to grow in vitro, and immortalized or cancer cell lines were the only models available. As discussed in this chapter, this new technique has revolutionized the field of intestinal transport physiology, pathology,

and pharmacology and has removed many of the limitations inherent to the traditional models. Human intestinal organoids are also unique in their capacity to allow personalized theratyping of drugs that could be applied in ion transport diseases such as CF and diarrheal disease. Because human viruses (e.g., rotavirus; norovirus) and microbes very often replicate poorly in transformed cell lines and animal models as a host, the availability of biobanked human organoids allows a thorough exploration of the infection mechanism and repetitive preclinical testing of novel antiviral or antimicrobial drugs (Saxena et al. 2016; Yin et al. 2015, 2016; Estes et al. 2019). Furthermore, because intestinal organoids have now been generated successfully from many different animal species, they facilitate a cross-species comparison of ion transport properties in well-defined intestinal segments and may provide a better insight into the evolution of intestinal ion transporters and their regulation (Schwarz et al. 2015).

Although the enteroids and colonoids discussed in this chapter remain reductionist models, i.e., are missing the complexity of the native tissue, this limitation can be overcome by coculturing the organoids with other cell types such as microbes, immune cells, and mesenchymal cells (Kim and Ingber 2013; In et al. 2016). Finally, peristalsis and fluid flow can be mimicked in a microfluidic gut-on-a-chip device (Kim et al. 2016; Bein et al. 2018; Shin et al. 2019). By integrating multiple electrode pairs in the chip, impedance measurements can be carried out to measure barrier function and differentiation status of the 2D organoids (van der Helm et al. 2019). Such transepithelial impedance measurements could also be exploited in future studies to analyze anion secretion, as shown previously for monolayers of Calu-3 airway cells (Tamada et al. 2001). This and other new developments illustrate that we stand only at the beginning of an era in which organoid technology will further expand and miniaturize, resulting in an unprecedented enrichment of knowledge of intestinal ion transport based on the analysis of near-physiological model systems.

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