Physiology in Health and Disease
Published on behalf of the American Physiological Society by Springer

Kirk L. Hamilton
Daniel C. Devor *Editors*

Ion Transport Across Epithelial Tissues and Disease

Ion Channels and Transporters of Epithelia in Health and Disease - Vol. 2

Second Edition

american physiological society

Springer

Physiology in Health and Disease

Published on behalf of the American Physiological Society by Springer

Physiology in Health and Disease

This book series is published on behalf of the American Physiological Society (APS) by Springer. Access to APS books published with Springer is free to APS members.
APS publishes three book series in partnership with Springer: *Physiology in Health and Disease* (formerly *Clinical Physiology*), *Methods in Physiology*, and *Perspectives in Physiology* (formerly *People and Ideas*), as well as general titles.

More information about this series at http://www.springer.com/series/11780

Kirk L. Hamilton • Daniel C. Devor
Editors

Ion Transport Across Epithelial Tissues and Disease

Ion Channels and Transporters of Epithelia in Health and Disease - Vol. 2

Second Edition

Springer

american physiological society

Editors
Kirk L. Hamilton
Department of Physiology, School of Biomedical Sciences
University of Otago
Dunedin, Otago, New Zealand

Daniel C. Devor
Department of Cell Biology
University of Pittsburgh
Pittsburgh, PA, USA

ISSN 2625-252X ISSN 2625-2538 (electronic)
Physiology in Health and Disease
ISBN 978-3-030-55309-8 ISBN 978-3-030-55310-4 (eBook)
https://doi.org/10.1007/978-3-030-55310-4

This Springer imprint is published by the registered company Springer Nature Switzerland AG.
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

We dedicate this second edition to our families . . . Judy, Nathan, and Emma for KLH, and Cathy, Caitlin, Emily, and Daniel for DCD.

Preface to Second Edition—Volume 2

Our ultimate goal for the first edition of *Ion Channels and Transporters of Epithelia in Health and Disease* was to provide a comprehensive and authoritative volume that encapsulated the most recent research findings in basic molecular physiology of epithelial ion channels and transporters of molecular diseases from the laboratory bench top to the bedside. Additionally, we envisioned that the book would be very exciting and useful to a range of readers from undergraduate and postgraduate students, to postdoctoral fellows, to research and clinical scientists providing a wealth of up-to-date research information in the field of epithelial ion channels and transporters in health and disease. We firmly believe that the first edition fulfilled a niche that was crucially required. We have been informed that the first edition of the book has proven to be the best performing APS/Springer book based on downloaded chapters, to date. This is a direct testament to the world-class scientists and clinicians who contributed excellent chapters to that edition. Of course, there were many epithelial ion channels and transporters which were not included in the first edition, but certainly warranted inclusion.

With our second edition, we have superseded our original expectations by increasing the number of chapters from 29 in the first edition to a three-volume second edition including 54 chapters, resulting in 25 new chapters. All of the original chapters have been expanded. Again, we were very fortunate to recruit "key" outstanding scientists and clinicians who contributed excellent chapters, some of whom were unable to commit to the first edition. In the end, the second edition has a total of 128 authors from 13 countries across four continents and both hemispheres. We truly believe that this book series represents a worldwide collaboration of outstanding international scientists and clinicians.

Volume 2: Ion Transport Across Epithelial Tissues and Disease

This is the second of three volumes highlighting the importance of epithelial ion channels and transporters in the basic physiology and pathophysiology of human diseases. For this volume, experts in their respective fields have contributed chapters on epithelial ion transport across a wide array of epithelial tissues and the use of organoids to study epithelial function. Volume 2 consists of 11 chapters (including 9 new chapters), which begins with a historical perspective of organoids as a model for intestinal ion transport physiology followed by two returning chapters focused on the roles of epithelia in the intestine and disease. These chapters are followed by new chapters on ion transport of the sweat gland, transporters of lactating mammary epithelia, lipid transport of the mammary gland, ion transport of the inner ear, retinal pigment epithelium, choroid plexus, and finally, transport function of ectoderm epithelial cells forming dental enamel. The chapters in this volume examine the overall epithelial physiology of various tissues of the body. In doing so, the authors inform the reader about the dynamic function of epithelial ion channels and transporters, in concert, to maintain normal cell physiology and changes in pathological states. This volume provides a holistic background for Volume 3 where chapters are focused on the background, structure of specific ion channels and transporters, and normal physiology and pathophysiology of disease.

It is our intent that the second edition continues to be the comprehensive and authoritative work that captures the recent research on the basic molecular physiology of epithelial ion channels and transporters of molecular diseases. We hope this new edition will be the "go-to" compendium that provides significant detailed research results about specific epithelial ion channels and transporters, and how these proteins play roles in molecular disease in epithelial tissues.

As stated in the preface of the first edition, the massive undertaking of a book of this enormity would certainly be an "Everest" of work. We want to sincerely thank all of our authors, and their families, who have spared time from their very busy work and non-work schedules to provide exciting and dynamic chapters, which provide depth of knowledge, informative description, and coverage of the basic physiology and pathophysiology of the topic of their individual chapters.

We want to, again, thank Dr. Dee Silverthorn who planted the "initial seed" that developed into the first edition, which stemmed from a Featured Topic session entitled "Ion Channels in Health and Disease" held during the Experimental Biology meetings in Boston in April 2013 (chaired by KLH). Then, based on the performance of that edition, Dee "twisted" our arms, with love, to attempt a second edition in 2017. We, once again, want to extend our huge thanks, gratitude, and appreciation to the members of the American Physiology Society Book Committee for their continued faith in us to pursue such a monumental second edition.

As with the first edition, this three-volume second edition would not have been possible without the excellent partnership between the American Physiological Society and Springer Nature and the publishing team in Heidelberg, Germany.

Many thanks to Markus Spaeth, Associate Editor (Life Science and Books), and Dr. Andrea Schlitzberger, Project Coordinator (Book Production Germany and Asia), who guided us on our second book publication journey never dreaming that this edition would be a three-volume book bonanza.

We extend special thanks to Anand Venkatachalam (Project Coordinator, Books, Chennai, India) at SPi Global who answered unending questions during the production process. We thank his production team who assisted us through the many stages of the publication of the second edition. We also thank Nancey Biswas (Project Management, SPi Content Solution, Puducherry, India), Nedounsejiane Narmadha (Production General, SPi Technologies, Puducherry, India), and Mahalakshmi Rajendran (Project Manager, SPi Technologies, Chennai, India) at SPi Global for their assistance for overseeing the production of the chapters during the final print and online file stages of the second edition.

We want to thank our mentors Douglas C. Eaton and the late Dale J. Benos for KLH; Michael E. Duffey and Raymond A. Frizzell for DCD; and our colleagues who guided us over the years to be able to undertake this book project.

Finally, and most importantly, we want to thank our families: Judy, Nathan, and Emma for KLH, and Cathy, Caitlin, Emily, and Daniel for DCD for all your love and support during this 8-year journey.

We dedicate this second edition to our families.

Dunedin, New Zealand — Kirk L. Hamilton
Pittsburgh, PA — Daniel C. Devor
July 2020

Preface

Ion channels and transporters play critical roles both in the homeostasis of normal function of the human body and during the disease process. Indeed, as of 2005, 16% of all Food and Drug Administration-approved drugs targeted ion channel and transporters, highlighting their importance in the disease process. Further, the Human Genome Project provided a wealth of genetic information that has since been utilized, and will again in the future, to describe the molecular pathophysiology of many human diseases. Over the recent years, our understanding of the pathophysiology of many diseases has been realized. The next great "step" is a combined scientific effort in basic, clinical, and pharmaceutical sciences to advance treatments of molecular diseases.

A number of unique ion channels and transporters are located within epithelial tissues of various organs including the kidney, intestine, pancreas, and respiratory tract, and all play crucial roles in various transport processes responsible for maintaining homeostasis. Ultimately, understanding the fundamentals of ion channels and transporters, in terms of function, modeling, regulation, molecular biology, trafficking, structure, and pharmacology, will shed light on the importance of ion channels and transporters in the basic physiology and pathophysiology of human diseases.

This book contains chapters written by notable world-leading scientists and clinicians in their respective research fields. The book consists of four sections. The first section of the book is entitled **Basic Epithelial Ion Transport Principles and Function** (Chapters 1–8) and spans the broad fundamentals of chloride, sodium, potassium, and bicarbonate transepithelial ion transport, the most recent developments in cell volume regulation, the mathematical modeling of these processes, the mechanisms by which these membrane proteins are correctly sorted to the apical and basolateral membranes, and protein folding of ion channels and transporters. The chapters in Section 1 provide the foundation of the molecular "participants" and epithelial cell models that play key roles in transepithelial ion transport function of epithelia detailed throughout the rest of this volume.

The second section is entitled **Epithelial Ion Channels and Transporters** and contains seventeen chapters (9–25) in which authors have concentrated their discussion on a particular ion channel or transporter ranging from chloride channels to the Na^+/K^+-ATPase, for example. Generally, the authors have initially provided a broad perspective of the physiology/biology of a particular ion channel or transporter in epithelial tissues, followed by a focused in-depth discussion of the latest physiology, cell biology, and molecular biology of the ion channel/transporter and then finish their discussion on aspects of pathophysiology and disease.

It will be appreciated following the discussion of the various ion channels and transporters that many of these transport proteins are potential pharmacological targets for possible treatment of disease. Therefore, the third section is entitled **Pharmacology of Potassium Channels** that consists of two chapters (26 and 27) that provide the latest developments on the pharmacology of calcium-activated potassium channels and small-molecule pharmacology of inward rectified potassium channels. It should be noted, however, that pharmacological information about various ion channels and transporters is also provided in some of the chapters found within Section 2 of this volume.

Finally, the last section in the book is entitled **Diseases in Epithelia** and consists of two chapters (28 and 29). These chapters are designed to bridge the basic cellular models and epithelial transport functions discussed throughout this volume with a compelling clinical perspective: from bench to bedside. In these chapters, Dr. Whitcomb discusses the role of ion channels and transporters in pancreatic disease, while Dr. Ameen and her colleagues similarly provide insights into the secretory diarrheas.

Our utmost goal, with this book, was to provide a comprehensive and authoritative volume that encapsulates the most recent research findings in the basic physiology of ion channels and transporters of molecular diseases from the laboratory bench top to the bedside. Additionally, we hope that the book will be very exciting and useful to a range of readers from students to research scientists providing a wealth of up-to-date research information in the field of epithelial ion channels and transporters in health and disease.

The undertaking of a book of this scale would always be a “mountain” of work. We want to give our heartfelt thanks to all of our authors who have taken time from their very busy work and non-work schedules to provide excellent chapters, which provided depth of knowledge, informative description, and coverage of the basic physiology and pathophysiology of the topic of their particular chapters.

We want to thank Dr. Dee Silverthorn who planted the “seed” that developed into this volume, which stemmed from a Featured Topic session entitled “Ion Channels in Health and Disease” held during the Experimental Biology meetings in Boston in April 2013 (chaired by KLH). We thank the members of the American Physiology Society (APS) Book Committee who had faith in us to pursue such an exciting book.

As with any book, this volume would not have been possible without the excellent partnership between the APS and Springer-Verlag and the publishing team at Heidelberg, Germany (Britta Mueller, Springer Editor, and Jutta Lindenborn, Project Coordinator). We wish to thank Portia Wong, our Developmental Editor at

Springer+Business Media (San Mateo, CA), and her team who assisted with the early stages of the publishing process that greatly added to this contribution. Finally, special thanks to Shanthi Ramamoorthy (Production Editor, Books) and Ramya Prakash (Project Manager) of Publishing—Springer, SPi Content Solutions—SPi Global and their production team who assisted us through the final stages of the publication of our book.

Finally, we want to thank our mentors Douglas C. Eaton and the late Dale J. Benos for KLH; Michael E. Duffey and Raymond A. Frizzell for DCD; and our colleagues who guided us over the years to be able to undertake this volume.

Dunedin, New Zealand — Kirk L. Hamilton
Pittsburgh, PA — Daniel C. Devor
June 2015

Contents

About the Editors

Kirk L. Hamilton was born in Baltimore, Maryland in 1953. He gained his undergraduate (biology/chemistry) and M.Sc. (ecology) degrees from the University of Texas at Arlington. He obtained his Ph.D. at Utah State University under the tutelage of Dr. James A. Gessaman, where he studied incubation physiology of barn owls. His first postdoctoral position was at the University of Texas Medical Branch in Galveston, Texas under the mentorship of Dr. Douglas C. Eaton where he studied epithelial ion transport, specifically, the epithelial sodium channel (ENaC). He then moved to the Department of Physiology at the University of Alabama, Birmingham for additional post-doctoral training under the supervision of the late Dr. Dale J. Benos where he further studied ENaC and nonspecific cation channels. He took his first academic post

in the Department of Biology at Xavier University of Louisiana in New Orleans (1990–1994). He then joined the Department of Physiology at the University of Otago in 1994 and he is currently an Associate Professor. He has focused his research on the molecular physiology and trafficking of potassium channels (specifically KCa3.1). He has published more than 60 papers and book chapters. His research work has been funded by the NIH, American Heart Association, Cystic Fibrosis Foundation, and Lottery Health Board New Zealand. Dr. Devor and he have been collaborators since 1999. When he is not working, he enjoys playing guitar (blues and jazz) and volleyball. Kirk is married to Judith Rodda, a recent Ph.D. graduate in spatial ecology. They have two children, Nathan (b. 1995) and Emma (b. 1998).

Daniel C. Devor was born in Vandercook Lake, Michigan in 1961. His education took him through Southampton College of Long Island University, where he studied Marine Biology, before entering SUNY Buffalo for his Ph.D., under the guidance of Dr. Michael E. Duffey. During this time, he studied the role of basolateral potassium channels in regulating transepithelial ion transport. He subsequently did his postdoctoral work at the University of Alabama, Birmingham, under the mentorship of Dr. Raymond A. Frizzell, where he studied both apical CFTR and basolateral KCa3.1

in intestinal and airway epithelia. He joined the University of Pittsburgh faculty in 1995 where he is currently a professor of cell biology. During this time, he has continued to study the regulation, gating, and trafficking of KCa3.1 as well as the related family member, KCa2.3, publishing more than 50 papers on these topics. These studies have been funded by the NIH, Cystic Fibrosis Foundation, American Heart Association and pharmaceutical industry. When not in the lab, he enjoys photography and growing exotic plants. Dan is married to Catherine Seluga, an elementary school teacher. They have three children, Caitlin (b. 1990), Emily (b. 1993) and Daniel (b. 1997).

Chapter 1
Organoids as a Model for Intestinal Ion Transport Physiology

Hugo R. de Jonge, Marcel J. C. Bijvelds, Ashlee M. Strubberg, Jinghua Liu, and Lane L. Clarke

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

H. R. de Jonge · M. J. C. Bijvelds
Department of Gastroenterology and Hepatology, Erasmus MC University Medical Center, Rotterdam, The Netherlands

A. M. Strubberg · L. L. Clarke (✉)
Dalton Cardiovascular Research Center, Columbia, MO, USA

Department of Biomedical Sciences, University of Missouri, Columbia, MO, USA
e-mail: ClarkeL@missouri.edu

J. Liu
Dalton Cardiovascular Research Center, Columbia, MO, USA

K. L. Hamilton, D. C. Devor (eds.), *Ion Transport Across Epithelial Tissues and Disease*, Physiology in Health and Disease,
https://doi.org/10.1007/978-3-030-55310-4_1

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

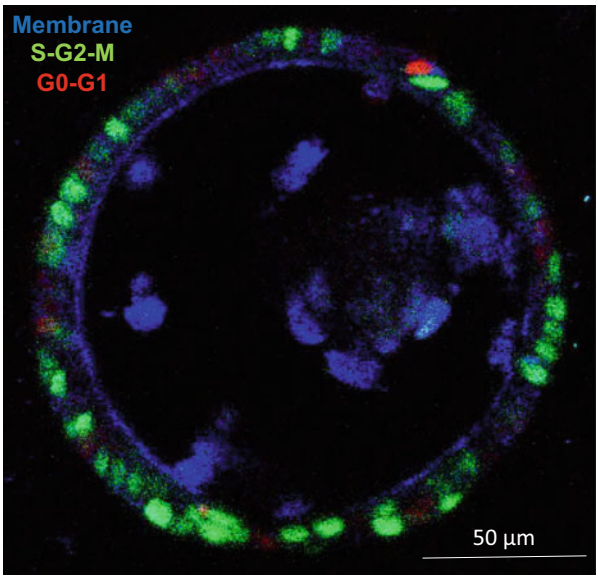

Fig. 1.1 FUCCI2 enterosphere. Optical cross-section of an intestinal stem cell-enriched enterosphere from a Fucci2 mouse intestine (Matsu-Ura et al. 2016), provided by RIKEN Center for Life Science Technologies (Kobe). Cell nuclei indicate quiescent cells (G0–G1 phases, red nuclei) and actively cycling 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

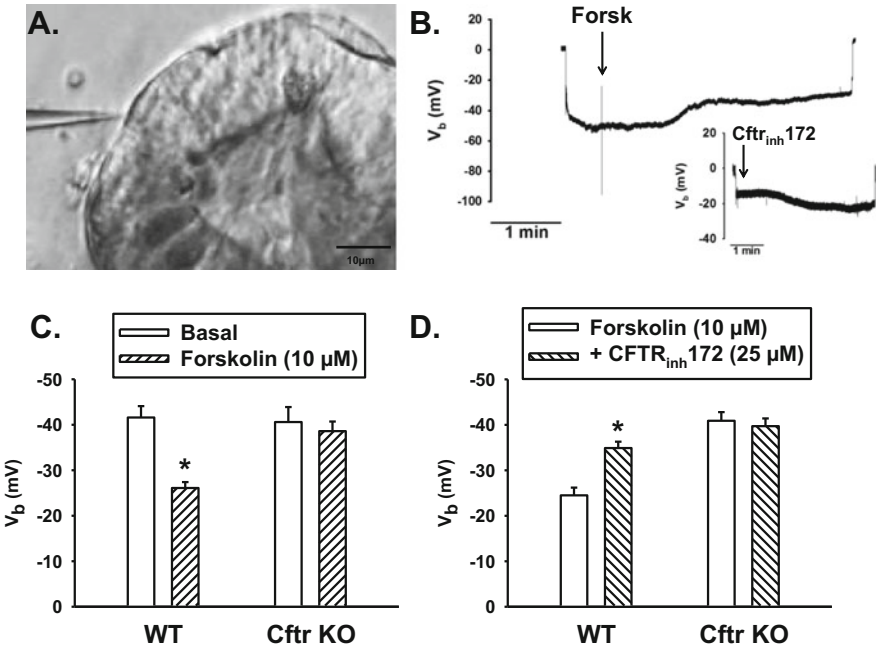

Fig. 1.2 Microelectrode analysis of Cftr-dependent changes in membrane potential in enteroid crypt epithelium. (**a**) Micrograph of enteroid crypt epithelial cell impaled with a conventional microelectrode (magnification: ×200). (**b**) Representative recording of the basolateral membrane potential (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

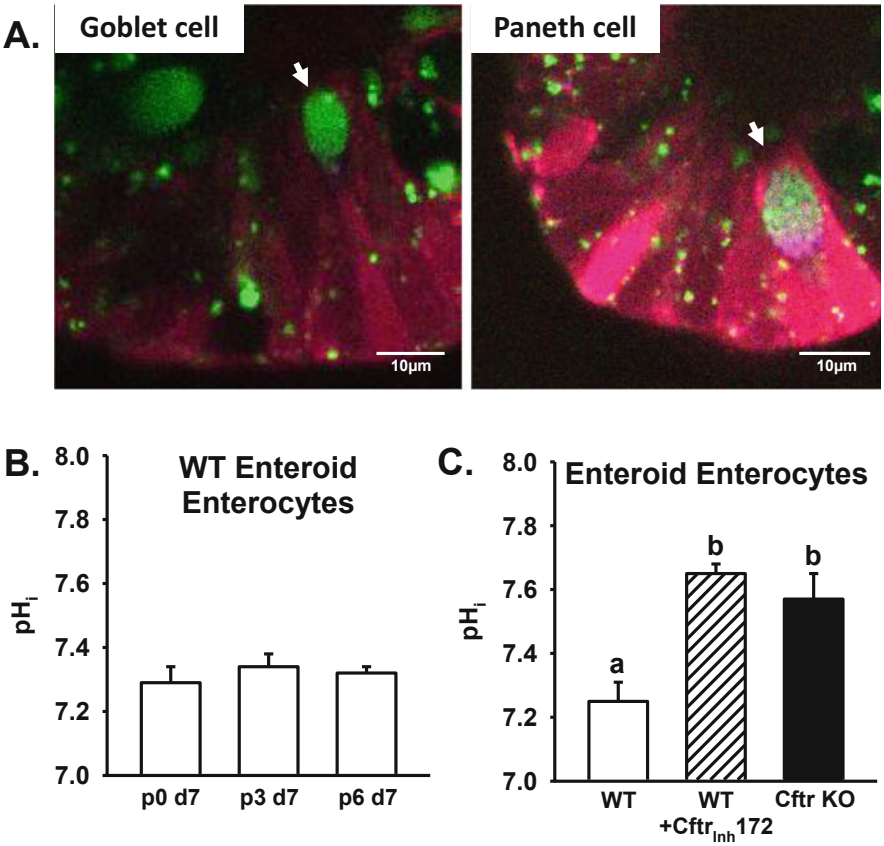

Fig. 1.3 Cftr-dependent effect on basal pH_i in enteroid crypt epithelium. (**a**) Merged confocal images of enteroid crypt epithelium stained with quinicrine (green) to identify granulated secretory cells (goblet and Paneth) and the pH-sensitive dye SNARF 5F (red) for measurement of pH_i (magnification: ×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.

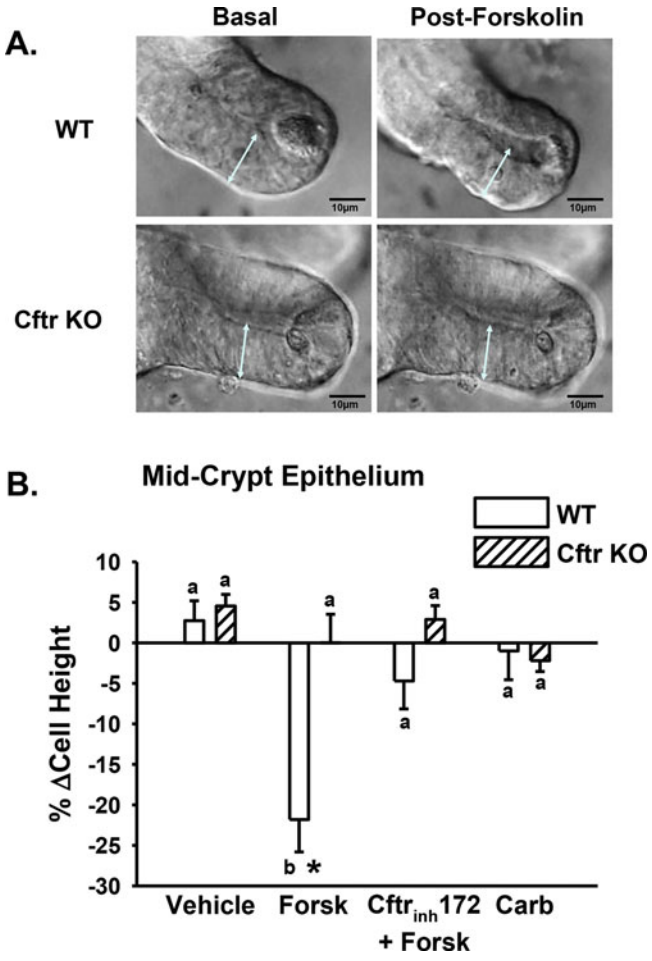

Fig. 1.4 Cftr-dependent cell shrinkage in enteroid crypts. (**a**) Photomicrographs of WT and Cftr KO enteroid crypts before (basal) and after 10-min exposure to 10 μM forskolin (post-forskolin). Arrowed bar indicates measurement of epithelial cell height as an index of cell shrinkage before forskolin 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

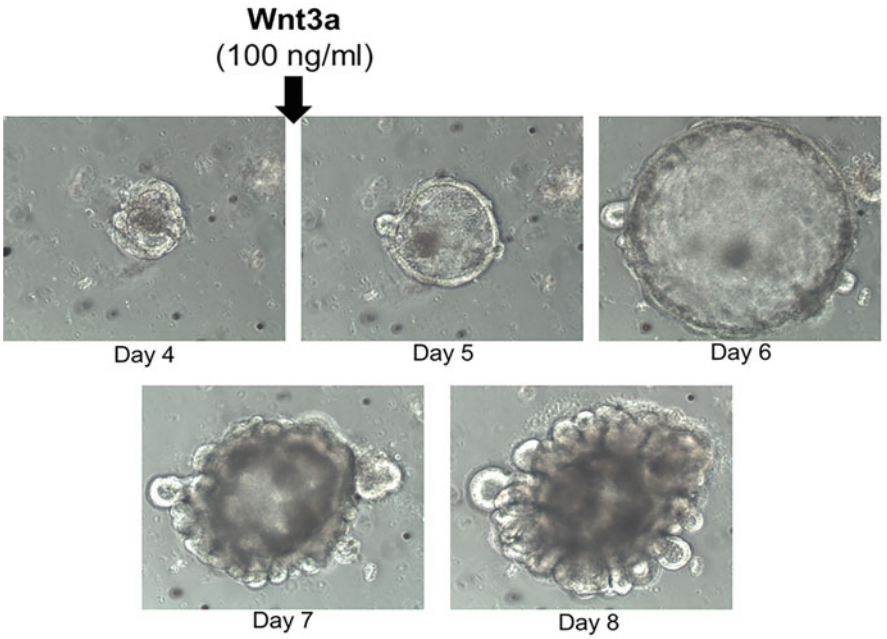

Fig. 1.5 Time course of WT enteroid treated with a single dose of Wnt3a (100 ng/mL on Day 4 in culture). Note expansion of organoid after 2 days (Day 6) and subsequent formation of multiple crypts (Day 8)

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

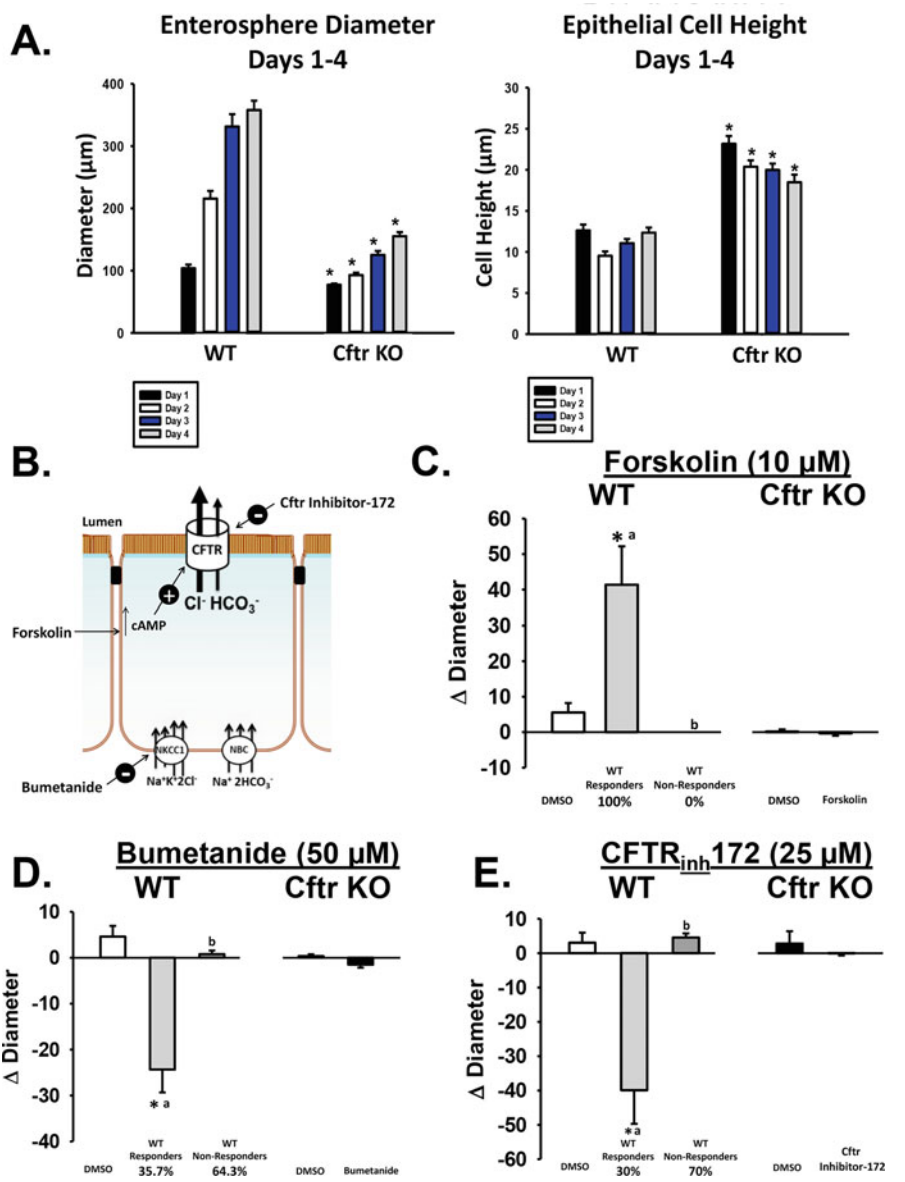

Fig. 1.6 Anion transport in murine enterospheres. (**a**) Time course of WT and Cftr KO enterospheres in culture showing enterosphere diameter and epithelial cell height. *$P < 0.001$ vs. WT, $n = 35$–42 enterospheres from 3 WT/Cftr KO sex-matched littermates. (**b**) Schematic representation of the 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

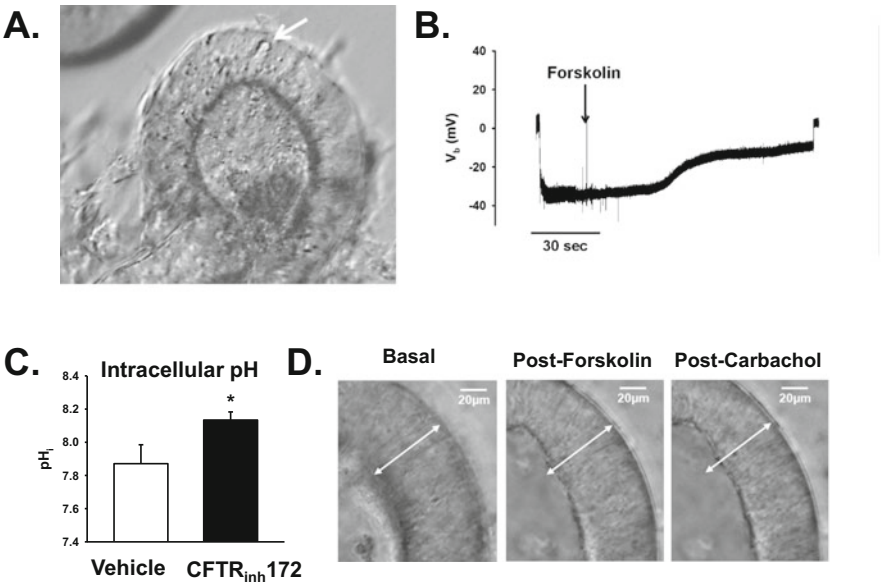

Fig. 1.7 Evidence of CFTR function in intestinal-differentiated iPSCs (HIOs). (**a**) Human intestinal organoid (HIO) from a non-CF, gut-differentiated iPSC (arrow, Paneth cell at crypt base). HIOs were a gift from Dr. James Wells, University of Cincinnati, Cincinnati Children's Medical Center. (**b**) 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