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The Contributions of Human Mini-Intestines to the Study of Intestinal Physiology and Pathophysiology

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Abstract

The lack of accessibility to normal and diseased human intestine and the inability to separate the different functional compartments of the intestine even when tissue could be obtained have held back the understanding of human intestinal physiology. Clevers and his associates identified intestinal stem cells and established conditions to grow “mini-intestines” ex vivo in differentiated and undifferentiated conditions. This pioneering work has made a new model of the human intestine available and has begun making contributions to the understanding of human intestinal transport in normal physiologic conditions and the pathophysiology of intestinal diseases. However, this model is reductionist and lacks many of the complexities of normal intestine. Consequently, it is not yet possible to predict how great the advances using this model will be for understanding human physiology and pathophysiology, nor how the model will be modified to include multiple other intestinal cell types and physical forces necessary to more closely approximate normal intestine. This review describes recent studies using mini-intestines, which have readdressed previously established models of normal intestinal transport physiology and newly examined intestinal pathophysiology. The emphasis is on studies with human enteroids grown either as three-dimensional spheroids or two-dimensional monolayers. In addition, comments are provided on mouse studies in cases when human studies have not yet been described.

Keywords

enteroids; organoids; intestinal physiology; transport; host pathogen

INTRODUCTION

The intestine digests and absorbs nutrients, with some specific functions carried out by the individual intestinal segments. Also, the intestine is the second human organ system

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involved in water and electrolyte homeostasis. Unlike the kidney, which can compensate for large changes in body fluid content, the capacity of the gastrointestinal (GI) tract to maintain hydration and volume homeostasis is often overwhelmed by pathophysiological processes, such as bacterial and viral diarrheal diseases, inflammatory diseases, and intestinal damage induced by ischemia or radiation.

Previously, two main intestinal models have been used to study normal human transport physiology and changes in diarrheal diseases. The first is transformed cancer-derived intestinal cell lines, which grow in a polarized manner and carry out vectorial transport involved in intestinal Na absorption and Cl and HCO₃ secretion. The commonly used cell lines include T84, Caco-2, and HT29 cells, which have been used to model the human intestine due to their ability to replicate intestinal permeability and membrane transport (1). However, they have several limitations. Sub-cloned cell lines used at different laboratories often vary in genetic status and biological behavior, such as growth rate, transepithelial resistance, metabolic activity, and the extent of expression of transport proteins (2). They also differ in functional and transport characteristics as their passage number increases. Other limitations include a lack of intestinal segment specificity and failure to mimic the complexity of the intestinal epithelium with each model containing only one of the cell types present in the intestine. Unlike in normal living intestine, cells grown under static conditions cannot be grown with living microbes for extended periods.

The second traditional intestinal model is animal intestine, which can be studied in intact animals or ex vivo for mechanistic evaluation. However, animal models are costly, labor-intensive, and often fail to predict human responses to therapy; they also poorly mimic human drug–drug interactions (3–8). Furthermore, they cannot be used in high-throughput applications, and it is not known how similar the contributions and segmental localization of transport proteins are to what is present in the human intestine.

These limitations of existing intestinal models have severely hampered the drug development process (9–11). For example, effective drugs to treat severe acute and chronic diarrheal diseases are lacking despite the need documented by the continued death from diarrhea of ~800,000 children per year in developing countries. Ironically, deodorized tincture of opium—initially described by Hippocrates in 400 BC and later modified by Paracelsus in the early sixteenth century—is still one of the most widely used effective medications for diarrhea.

For these reasons, it was desirable to develop an alternative model to study human intestinal function. The recent development of human “mini-intestines” by Clevers and coworkers (12–21) offers the unique potential to overcome many limitations of the previous models. These can also be applied to precision medicine, regenerative medicine, and tissue engineering. In this review, we focus on the development and application of human mini-intestines to better understand intestinal physiology, pathophysiology of host-pathogen interactions, and some genetic diseases.

DEVELOPMENT OF HUMAN MINI-INTESTINES

There are two types of mini-intestines: Those derived from intestinal stem cells are called enteroids, and those derived from iPS cells are called organoids. The Intestinal Stem Cell Consortium proposed these terms, which allows them to be considered separately and which is important when considering the strengths and weaknesses of both preparations (22).

Enteroids

Human enteroids/colonoids encompass a general term for mini-intestines grown either from small intestinal stem cells (enteroids) or colonic stem cells (colonoids). The terms human enteroids refers to both enteroids and colonoids in this review. Ex vivo primary cultures of human enteroids are usually obtained from biopsies or surgical resections of intestinal tissue. It was thought that mini-intestines could be produced by a single LGR5⁺-Paneth cell pair. Currently, mini-intestines made from intestinal stem cells do not start from such single pairs, but it is unclear whether the enteroids created by the two aforementioned approaches differ. The initial development of these organotypic cultures and the characterization of the LGR5⁺ stem cells were previously described in many other reviews (23–28). The human enteroid and colonoid cultures contain only epithelial cells and can proliferate in the absence of mesenchyme. The growth factors needed to create a stem cell niche are provided in the media by various recombinant proteins, conditioned media, and inhibitors (29–31). Of these growth factors, WNT3A is the most essential to sustain proliferation and maintain “stemness” (19, 29, 31, 32). In high WNT3A conditions, the enteroid/colonoid cultures are characterized as “undifferentiated” with a crypt-like protein profile in which a majority of the cells are in a proliferative state. The cells present are the LGR5⁺ stem cell, Paneth cells, and the proliferating transit amplifying cells. Removal of WNT3A results in the loss of stemness and proliferation leading to differentiation of the LGR5⁺ stem cells to terminally differentiated small intestinal villus-like and colonic surface cells, including enterocytes, goblet cells, enteroendocrine cells, and Tuft cells; further manipulations allow the production of M cells. Various structural changes accompany differentiation of the cultures, including the formation of dense microvilli with uniform height; increased mucus production and a thick glycocalyx; expression of villus brush border enzymes (lactase, sucrase-isomaltase, alkaline phosphatase); and microvillar bridges that include protocadherin-24 (33, 34).

Mouse enteroids endogenously produce large amounts of Wnt and thus do not have the same need for exogenous Wnt required in long-term human enteroid culture (35). This probably explains why it has not been possible to separate clearly undifferentiated from differentiated enteroid populations in the mouse by removing exogenous Wnt. However, mouse enteroids, which are much more readily available than human enteroids, are technically less challenging, less time-consuming, and can be genetically modified in lineage tracing studies. Thus, much of the understanding of intestinal stem cell biology has been derived from studies of mouse enteroids.

Organoids

Human organoids, in contrast to enteroids, are derived from induced pluripotent stem cells (iPSC) or embryonic stem cells (ESCs) (36, 37). Wells et al. (38) pioneered the generation of human intestinal organoid cultures. Briefly, recombinant activin A initiates *Sox17* expression and leads to posterior endoderm patterning into a primitive gut, which gives rise to three-dimensional (3D) spheroids representing fetal small intestine. The organoids remain fetal-like and, thus far, conditions to induce intestinal segment specificity have not been determined (39). Similar to the adult-derived enteroids, the growth of these organoids is also WNT3A dependent. They are composed of both crypt- and surface-residing intestinal cells with a surrounding mesenchyme. The mesenchyme consists of myofibroblasts, smooth muscle, and some endothelial cells. Although fetal-like, the organoids form a pseudovillus structure due to smooth muscle contraction. When grown under the capsule of the mouse kidney, these organoids greatly enlarge and exhibit a slightly more mature intestinal signature, which includes expression of both brush border digestive enzymes and defensins, which are intestinal antimicrobial peptides (40). These larger organoids can be removed and used for crypt isolation and enteroid formation.

Human organoids provide a distinct advantage in developmental studies of the intestine in which fetal- to adult-related changes can be documented (41). Current studies are underway to incorporate enteric nerves and to differentiate organoids into specific segments of the small intestine and colon based on alteration of the growth factors used.

Differentiation Versus Maturation

Regarding the cellular identities of the intestinal enteroid and organoid cultures, there is a subtle difference between differentiation and maturation of the cells in these cultures. As described previously (37, 42), differentiation denotes the transition from pluripotent (organoid) or multipotent (enteroid) stem cells to transit-amplifying cells, then to a terminally differentiated intestinal cell, such as goblet cells or enteroendocrine cells. Organoid or enteroid cultures that are differentiated have lost their proliferation ability and stemness but may not be fully mature. Maturation denotes cells that are committed to a specific lineage and are somewhere in development to the final mature cell (43, 44). For example, to form a goblet cell, stem cells in the *in vivo* intestine will differentiate to precursor cells that begin to show signs of mucous granules but do not yet express the full protein signature of a functional goblet cell (45). Over time, these precursor cells will mature to become a terminally differentiated and mature goblet cell.

HUMAN MINI-INTESTINES IN THE STUDY OF INTESTINAL PHYSIOLOGY

Human enteroids potentially represent a model of normal human intestine that exists as a cell line that can be reproducibly studied over time and can be differentiated in a way to create separate villus-like and crypt-like cell populations (33). Human enteroids, which contain only epithelial cells, can be grown as 3Dspheroids and 2Dmonolayers from each segment of the entire intestine—from the duodenum to the rectum. The structural and functional characterization of this model suggests similarities to multiple aspects of normal intestine, although few detailed studies have been reported.

Crypt–Villus Development

The normal intestinal vertical axis includes tall villi, each formed from 6–10 crypts (46). Whereas differentiated enteroids have many characteristics of mature villus enterocytes, they do not form villi despite attempts to grow them on scaffolds that artificially create crypt and villus compartments (47). Organoids also lack villi when grown in 3D form. However, when buried under the kidney capsule or in the omentum of the mouse, the organoids attach to the mouse vessels, greatly increase in size, and form a crypt-villus axis. This is the only condition in which mini-intestines have been induced to develop villus structures (39, 40).

Segmental Specificity

In addition to differentiation along the crypt-villus axis, human intestine exhibits regional identity along the cephalocaudal axis. Several transcription factors, including GATA4 and CDX2, control the expression of genes in the different intestinal segments (48, 49). GATA4 is expressed in duodenum and jejunum where it inhibits expression of ileal-specific genes (48), whereas CDX2 is expressed throughout small and large intestine and has an important role in the formation of normal intestinal identity (49).

Each intestinal segment has some highly specialized functions, which are associated with multiple proteins that are differentially expressed along the intestinal horizontal axis. For example, duodenal cytochrome b reductase 1 (CYBRD1) is expressed only in the duodenum where it is involved in iron metabolism (50); lactase phlorizin hydrolase (LCT) is mainly expressed in the jejunum (51); and Na-bile acid transporter (SLC10A2) is expressed apically on enterocytes exclusively in the ileum where it takes up bile acids from the intestinal lumen (52).

Enteroids maintain intestinal segmental specificity that parallels distinct protein expression in intact intestine. This segmental specificity is stably maintained over multiple passages at both the gene and protein expression levels. For example, mRNA sequencing and qRT-PCR of enteroid cultures showed that GATA4, CYBRD1, and LCT were expressed in duodenum and jejunum but not in ileum, whereas SLC10A2 was expressed in ileum but not in duodenum or jejunum, and that expression was maintained throughout long-term cultures (53). When the culture media was switched to differentiation media (WNT withdrawal), GATA4 expression levels did not change, whereas CYBRD1 had equal or even higher levels in ileal enteroids compared to duodenal enteroids. LCT was induced in both duodenum and ileum, but ileal expression was rather low. In contrast, the ileal marker SLC10A2 was highly restricted to differentiated enteroids derived from ileum. These data indicate that WNT signaling inhibits the expression of genes involved in the function of differentiated cells (CYBRD1, LCT, SLC10A2) but not those involved in spatial patterning (GATA4). Furthermore, because all enteroids were exposed to the same extracellular factors, it is tempting to conclude that the functional fate of human differentiated cell types is intrinsically programmed within the segment-specific stem cell niche, independent of location-specific external signals from mesenchyme or microbiota. Thus, using enteroids has the potential to recapitulate diseases that afflict a specific segment of the intestine. The mechanisms of segment-specific gene and protein expression involve transcription factors,

but the role of the epigenetic profile of each intestinal segment and its preservation in segment-specific enteroids has not yet been described.

Stemness

The lifespan of the intestinal epithelium is 5–7 days, and it is the fastest regenerating organ system in the body. Actively dividing stem cells are located in the intestinal crypt. They undergo division, which is debated as being asymmetrical or symmetrical, for self-renewal and generation of undifferentiated progenitor cells that give rise to the absorptive and secretory cell lineages that define the crypt–villus axis (21). Based on lineage tracing studies in mice, the renewal of the intestinal epithelium is attributed to the stem cell population that expresses Lgr5. However, information related to human studies is still largely lacking due to difficulty identifying LGR5⁺ cells in human intestine; this is probably due to the lack of appropriate antibodies and the low expression of LGR5. Alternate approaches, such as the use of SmartFlare RNA-based technology, are now being developed (54). To test the hypothesis that Lgr5⁺ stem cells are responsible for epithelial regeneration, several mouse models were developed to determine if an alternative stem cell population can replenish the intestinal epithelium under conditions in which Lgr5⁺ stem cells are ablated or damaged. These studies demonstrate that upon radiation-induced injury or Lgr5⁺ stem cell ablation, alternative stem cell populations become actively dividing stem cells that are or become Lgr5⁺ (55). This suggests that Lgr5 positivity characterizes the stem cells required to regenerate the intestinal epithelium. Furthermore, clonal lineage-tracing studies by the Clevers group (12) demonstrated that a single Lgr5⁺ stem cell (out of ~15/crypt) can dominate the production of progenitor cells along a region of the crypt–villus axis. The decision on which Lgr5⁺ cells become predominant appears based on the amount of WNT provided by contiguous Paneth cells (56) or nearby mesenchymal cells, especially myofibroblasts (57). These murine studies are supported by in vitro enteroid culture studies. In the absence of Wnt and R-spondin (the ligand for the receptor Lgr5), enteroids fail to develop in 3D culture (18, 20), thus demonstrating the dependence of Wnt and R-spondin to maintain stemness. Although Wnt and R-spondin are also required for human enteroid growth, future studies are needed to test whether alternative stem cell populations also convert to LGR5⁺ cells and regenerate the intestinal epithelium under similar stem cell depleted or damaged conditions in human enteroids.

Plasticity

The ability of a cell to adopt an alternate identity in response to internal or external challenge is referred to as plasticity. Differentiated progenitor cells can convert lineages to become different type of progenitors by a process termed transdifferentiation. Alternatively, a differentiated cell may become less differentiated or dedifferentiated and have the capacity to proliferate. Evidence of plasticity has been documented in epithelia of the intestine, kidney, liver, lungs, and adrenal glands. In the intestine, progenitor cells of the secretory and absorptive cell lineages can dedifferentiate into Lgr5⁺ stem cells (55). Furthermore, these cells have the capacity to regenerate the intestinal epithelium after injury as well as to form propagating enteroid cultures in vitro (55). Whether secretory and absorptive progenitor cell precursors can dedifferentiate in the human intestinal epithelium has not yet been studied. The potential for epithelial regeneration from differentiated cell types may provide insight

into developing novel therapeutic regenerative medicine strategies to replace lost epithelia and organs.

Impact of Physical Forces

The intestinal mucosa is subjected to multiple physical forces during normal gut function, including shear stress from the basolateral blood flow and luminal flow of ~9 L per day, deformation from peristaltic muscle contractions, and strain associated with rhythmic villus “pumping.” These forces contribute to the rate of proliferation and maturation of the intact intestine. For instance, in vivo stretch of pig jejunum with an intraluminal device for seven days stimulated epithelial growth and proliferation, while maintaining epithelial barrier and absorptive functions (58). In vitro strain of a physiologically relevant amplitude and frequency promotes proliferation and modulates differentiation in Caco-2 monolayers (59) and nontransformed primary human enterocytes isolated from surgical specimens (60).

Although most studies were done with enteroids grown in the 3D spheroid form, the ability to grow them as 2D monolayers was recently achieved (30, 34) (Figure 1*a*). This was accomplished by the initial application of extracellular matrix (ECM) to semipermeable platforms such as Transwell inserts. The extracellular matrix used in monolayer development does not appear to be specific, with collagen IV being the most commonly used (34).

Enteroids grown as monolayers can be exposed to mimics of the physical forces to which the normal intestine is exposed. The Ingber group (61–63) recently described a “human gut-on-a-chip” microdevice with two closely apposed microfluidic channels separated by a thin porous membrane coated with ECM on which polarized intestinal epithelial cells could be grown. The mechanically active gut microenvironment was recreated by flowing culture medium through the apical and basolateral channels at a low rate (30–60 $\mu\text{L}/\text{h}$, equivalent to 0.02–0.04 dyne/cm^2 shear stress) and by repeated suction to the side vacuum channels to exert rhythmic mechanical strain (10%; 0.15 Hz) to mimic peristaltic contractions. These forces were designed to resemble levels experienced by epithelial cells in living intestine. This model has not yet been reported with normal human enteroids grown as monolayers but was applied to Caco-2 cells. Compared to static culture in Transwell filters, Caco-2 cells cultured under stretch and flow conditions demonstrated faster polarization and differentiation, increased cell height, enhanced barrier function with higher transepithelial resistance, and increased expression of drug-metabolizing cytochrome P450 (CYP3A4) activity, which plays a central role in first-pass metabolism of drugs in human intestine (62, 64). Most surprisingly, the Caco-2 cells exposed to these physical forces spontaneously produced all four types of intestinal epithelial cells (absorptive, goblet, enteroendocrine, and Paneth cells), which does not occur in static Transwell cultures. However, the physical forces modeled so far recapitulate only the minimal set of forces that the intestine is normally exposed to; many forces are not adequately characterized in normal human subjects. Also, in vivo intestinal peristaltic motions are not as rhythmic or linear as those in the currently engineered models. Furthermore, villi undergo pumping, such as up and down motions, which have not been incorporated into the current microfluidic devices. Application of physical forces (fluid flow and strain) to enteroids grown as monolayers will

add another dimension to allow closer modeling of normal intestinal function. Advances in this aspect of enteroid biology require close interactions between biomedical engineers and biologists.

Epithelial Transport Physiology

Human enteroids are used to reexamine normal human transport physiology. Multiple gaps remain in understanding the proteins that contribute to intestinal electrolyte transport under normal digestive conditions. These include the definition of the transporters involved in intestinal Na and Cl absorption and anion secretion, their distribution along the vertical and horizontal axes of the intestine, the coordination of their functions, and their regulation during normal digestive physiology as well as during acute and chronic diarrheal diseases (65, 66). Whereas studies of epithelial transport using 3D human enteroids are just beginning, and studies with 2D monolayers are not described in detail, these approaches are likely to help reduce the gaps in knowledge (30, 33, 34).

The historical view of intestinal Na absorptive and anion secretory epithelial cells is that they are largely separate populations, with the former in the villus of the small intestine and surface and upper crypt of the colon and the latter being restricted to the crypts (Figure 2*a, b*) (65–67). The view is supported indirectly by several lines of evidence: (*a*) By placing oil over the intestine and inducing secretion with prostaglandin E₂ (PGE₂), fluid droplets representing fluid secretion occurred only over the crypts; (*b*) When rabbit jejunal crypts but not villus cells were damaged with cycloheximide, there was reduced cholera toxin related secretion without a change in D-glucose absorption; and (*c*) Exposing rabbit jejunum to hyperosmotic sulfate damaged the villus but not the crypt compartment and reduced D-glucose absorption, but it did not alter cholera toxin-induced fluid secretion (68–75). However, the historical view was challenged based on additional animal studies (71–75). In addition, Field (75) questioned the concept of crypt secretory cells migrating up the villus and converting into strictly absorptive cells because it required a large number of changes in transport protein expression to allow this conversion. Specifically, epithelial cells would need to suppress production of NKCC1, CFTR, and calcium-activated Cl channels to eliminate anion secretion and initiate production of NHE3 and DRA to allow neutral NaCl absorption.

Recent enteroid studies suggest some potential modifications of that dogma. Using a combination of qRT-PCR, immunoblotting, and immunostaining of differentiated versus undifferentiated human enteroids, we found the following (33; JY Yin, CM Tse, L Avula, unpublished data). First, NHE3, DRA, and CFTR were present in both differentiated and undifferentiated duodenal and jejunal enteroids. Second, there were moderate increases in NHE3 and DRA expression with differentiation (1.5–3 ×), whereas CFTR expression did not change significantly. The major changes observed were a marked reduction of NKCC1 expression with differentiation and a decrease in expression in several basolateral K channels. Third, functionally, both differentiated and undifferentiated 3D proximal small intestinal enteroids exhibited NHE3 and DRA activities with small increases in NHE3 activity with differentiation. Moreover, both differentiated and undifferentiated enteroids had forskolin-stimulated anion secretion. Quantitation by the forskolin-induced swelling (FIS)

assay demonstrated that the rate of swelling in undifferentiated jejunal enteroids exceeded that in differentiated enteroids (J. Foulke-Abel & M Donowitz, unpublished data). When human jejunal enteroids were grown as 2D monolayers, active ion secretion could be characterized via the Ussing chamber/voltage clamp technique. We found that differentiated and undifferentiated enteroids both exhibited active anion secretion induced by forskolin and carbachol, again with greater secretion rates in the undifferentiated enteroids (Figure 1*b*). We interpreted our results as an indication that neutral NaCl absorption and anion secretion occur in both villus-like differentiated and crypt-like undifferentiated enteroids (Figure 2*c*). Our data suggest that restriction of Na absorption to villus and colonic surface and upper crypt cells and anion secretion to crypt cells is not as rigid as initially believed (33).

These initial studies have raised further questions concerning the transport processes present both along the vertical and horizontal axes of the intestine: (*a*) If there is Cl secretion in the villus that lacks NKCC1, what is the basolateral Cl loader? (*b*) What is the contribution of villus anion secretion to Cl versus HCO₃ secretion, and how does this change in diarrheal diseases? (*c*) Are there differences in the transport proteins involved in HCO₃ secretion in villus versus crypt cells? (*d*) Does calcium-activated Cl secretion occur via intestinal anion channels? What is the molecular identity of those channels, and are they present in both the villus and crypt? (*e*) DRA, the SLC26 gene family member (A3), was suggested as taking part in both neutral NaCl absorption and HCO₃ secretion (76–81). The former is inhibited in cyclic AMP (cAMP)-related diarrheas, whereas the latter appears to be increased by cAMP. If both Na absorption and anion secretion occur in both villus and crypt cells, how does cAMP regulate DRA to allow both these changes in transport to occur? (*f*) Where in the normal intestine does neutral NaCl absorption occur? This process is made up of NHE3 linked to DRA (77, 82). In *in vivo* triple lumen perfusion studies in normal volunteers, proximal jejunum appeared to have apical Na/H exchange but little apical Cl/HCO₃ exchange, whereas both were present in the ileum (83, 84). Also, neutral NaCl absorption in the ileum was greater than in the jejunum (85). However, these results cannot be interpreted adequately at the current time, as the definition of the DRA distribution in normal human intestine has not been reported in detail. Examination of the distribution of neutral NaCl absorption at a functional level—which is important for normal intestinal Na absorption and which becomes abnormal in most diarrheal diseases—is also needed. Although these questions remain unaddressed, we posit that human enteroids grown as monolayers have the potential to help clarify these and other aspects of human intestinal transport physiology.

HUMAN MINI-INTESTINES IN THE STUDY OF INTESTINAL PATHOPHYSIOLOGY

Initial studies using human mini-intestines to examine gastrointestinal diseases have taken two forms: (*a*) Human enteroids from normal subjects are exposed to pathogenic factors that cause intestinal disorders, and (*b*) human enteroids from patients with intestinal disorders reproduce at least certain aspects of the disorder, indicating that the intestinal stem cells have been altered genetically or epigenetically. As examples of the second type of study, enteroids generated from patients with cystic fibrosis (CF) and microvillus inclusion disease exhibit previously described intestinal epithelial abnormalities (86–86; J. Goldenring, N. Zachos,

unpublished data). Moreover, a ~90% similarity in somatic mutations was observed in human colonoids generated from metastatic tumors (89). Based on these observations, human enteroids may be considered as translational models of the intestinal epithelium that are suited for “personalized or precision medicine” studies. Both approaches to using human mini-intestines to study GI diseases provide relevant human models that can be used not only to understand pathogenesis but also to identify, develop, and optimize novel therapies. Furthermore, enteroids generated from patient cohorts should prove valuable in the evaluation of drug efficacy, bioavailability, and toxicity as a preclinical testing platform. If human mini-intestines maintain characteristics of a specific GI disease, they can make major mechanistic and therapeutic contributions in the diseases with identified changes of specific protein expression. Inflammatory bowel disease (IBD) is such an example, but no significant descriptions of studies with human enteroids on this disease have been reported. Moreover, it is not known how much of the IBD phenotype is maintained in enteroid cultures.

Host–Pathogen Interactions

The intestine has an important barrier function in preventing direct contact with bacteria that reside in the lumen. One of the major areas examined by the use of enteroids is the study of the pathogenesis of infectious diarrhea. Human enteroids and organoids are exploited to study several different pathogens that cause human diarrheal diseases, including rotavirus, enterohemorrhagic *Escherichia coli* (EHEC), *Clostridium difficile*, and *Salmonella typhimurium*. Currently, several research groups, including ours, are using human enteroid and colonoid monolayers to study host–pathogen interactions (33, 34, 90). These studies have used enteroids grown as monolayers on Transwell filters and provide full access to apical and basolateral sides of the epithelium. This allows luminal exposure to pathogens and commensal bacteria, as occurs in the human intestine, and also permits apical and basolateral drug exposure. Also, by growing the enteroids as either undifferentiated crypt-like or differentiated villus/surface-like cells, effects on villus and crypt enterocyte populations can be examined separately (33, 34, 90). Current data suggest that human enteroids provide a useful system for interrogating some of the complex interactions between the enteric pathogens and the intestinal epithelium of the human host. Examples of the information learned so far using these models are presented below.

Rotavirus

Understanding the pathophysiology of rotavirus infection has increased by the use of iPSC-derived organoids and human enteroids (30, 91–93). Findings in 3D human enteroids were consistent with the known rotavirus targets in the intestine, including that rotavirus targets villus enterocytes and differentiated enteroids and replicates for up to 96 h, during which it produces an infectious virus (91, 92). The species specificity of rotavirus was also confirmed, as the percentage of human enterocytes infected with human rotavirus was several times that which occurred with simian rotavirus (92); and human rotavirus does not replicate in mouse intestinal epithelial cells in vivo and ex vivo (92, 93). In addition to infecting mature enterocytes, human rotavirus was also detected in enteroendocrine cells (92). This finding is consistent with a proposed model in which rotavirus stimulates serotonin production/release from enteroendocrine cells to activate the enteric nervous system. In fact, in a cat model of rotavirus, 50% of the diarrhea was neurally mediated (94).

Human enteroids are used to assess altered intestinal ion/water transport following exposure to the rotavirus enterotoxin, NSP4. Rotavirus pathophysiology involves NSP4, a viroporin, which induces Ca^{2+} -dependent luminal dilation of human enteroids as well as inhibition of NHE3. Inhibition of intestinal Na absorption appears to be a significant contributor to rotavirus-induced diarrhea (95). Because the pathophysiology of rotavirus diarrhea remains poorly understood, the recent technological advance of growing enteroids as monolayers, in addition to the already described 3D assays, should provide additional mechanistic insights. It would also allow identification of the ion/water transporters affected in rotavirus infection and clarify whether the changes occur only in the villus compartment or if they also involve the crypt.

Clostridium difficile—*C. difficile* colitis is the most frequent cause of hospital-acquired diarrhea and is responsible for increasing mortality in the United States. *C. difficile* produces two enterotoxins (Toxins A and B), both of which act by altering the small GTPase Rho (96, 97). Microinjection of *C. difficile* in human iPSC-derived organoids was used to model the pathophysiology of *C. difficile* colitis (note that colonic specificity of this disease could not be examined with this model) (98). *C. difficile* persisted for at least 12 h in the spheroid lumen and disrupted the tight junction function to increase permeability. In the enteroids, this was related to the production of *C. difficile* Toxin A but not Toxin B. *C. difficile* injection inhibited Mucin2 (MUC2) production, a main component of the colonic inner mucus layer, which plays a protective role against commensal and pathogenic bacteria. *C. difficile* also downregulated NHE3, an effect that occurred at least partially transcriptionally, possibly contributing to *C. difficile*-induced diarrhea. These results are also compatible with and might explain the high Na concentration in the stools of *C. difficile* colitis patients (99, 100). Although informative, these studies do not totally support the current understanding of the pathophysiology of *C. difficile* colitis in which Toxin B is also currently believed to contribute to the pathophysiology (101–103). Further studies are awaited comparing the effects of *C. difficile* and its toxins for effects on differentiated and undifferentiated human colonoids grown as monolayers.

Salmonellae—*Salmonellae* constitute a huge disease burden in the world, with over 90 million cases of gastroenteritis and 22 million typhoid cases every year (104). Human organoids have been used to explore the interaction of *S. typhimurium* with the human intestinal epithelium (105). After microinjection into the lumen of organoids, *Salmonellae* could invade the intestinal mucosal surface, with many bacteria residing within structures that resemble the described Salmonella-containing vacuoles. Also, imaging and RNA sequencing detected changes in transcriptional signatures, including an altered cytokine expression pattern, after the exposure of human organoids to the *Salmonellae*.

Enterohemorrhagic *Escherichia coli*

Every year, EHEC causes over 70,000 episodes of food-borne diarrhea in the United States. Due to the initial asymptomatic phase of this disease and the lack of a suitable animal model, the early sequence of events that precedes life-threatening hemorrhagic colitis and the EHEC-induced hemolytic uremic syndrome is not fully understood. Recent studies of EHEC using human colonoid monolayers demonstrate the interaction between Shiga toxin-

producing EHEC and the human colonic epithelium, a feat not achieved in whole animal models (106, 107). Differentiated colonoid monolayers produce a thick apical (>50 μm) attached mucus layer, which is made mainly of MUC2, similar to that present in intact human colon (108, 109). This suggests that colonoids might be a physiologically relevant model for EHEC infection. Using this model, we showed that EHEC destroys the inner colonic mucus layer, using the mucus as a high energy substrate for colonization and possibly as an initial anchor (34, 110). In addition, the EHEC serine protease EspP, which is secreted into the intestinal lumen after colonization, initiates brush border damage through targeting protocadherin-24, a building block of intermicrovillar bridges.

Congenital Diarrheal Diseases

This series of rare but devastating disorders either leads to early death or long-term dependence of the patients on intravenous hydration and central peripheral nutrition. A growing number of congenital diarrheal diseases are being identified in which specific diagnoses have led to definitive therapies and several cures (111–114). These disorders now include immunologic abnormalities; abnormalities of the adhesion molecule EpCAM (Tufting enteropathy); abnormalities in apical membrane trafficking (microvillus inclusion disease); abnormalities in genes needed for expression of subpopulations of epithelial cells (neurogenin-3 gene deficiency, which leads to a lack of enteroendocrine cells); functional or structural abnormalities of transport proteins, including SGLT1, DRA, and NHE3 (115); and changes in signaling molecules, for example, the activation of guanylyl cyclase C (137).

The use of human mini-intestines to study the pathogenesis of these disorders includes research on microvillus inclusion diseases (116, 117; J. Goldenring, N. Zachos, unpublished data). The most common etiology of microvillus inclusion disease is mutations in myosin VB (MyoVb) that lead to abnormalities in apical trafficking of structural brush border proteins as well as apical transporters. This disease is also associated with what is believed to be secondary abnormal endocytosis that gives rise to the characteristic microvillus inclusions, which are postulated as not being critical to the pathophysiology of the disease. Enteroids from MyoVb knockout mice exhibit a similar phenotype to the intact intestinal epithelium, including microvillus inclusions and intracellular alkaline phosphatase (116). Functionally, luminal swelling assays demonstrated an attenuated response by CFTR to forskolin in MyoVb knockout enteroids (116). Mutations in other proteins produce a phenotype similar to microvillus inclusion disease. For instance, a mutation in syntaxin 3 has been associated with a clinically similar disorder (118).

Tetratricopeptide Repeat Domain Protein 7A (*TTC7A*) Mutations

Mutation in *TTC7A* is associated with intestinal atresia with combined immunodeficiency (119–121). Due to the increased inflammation, this is called a form of IBD (120). In these patients, mucosal architecture is altered from the stomach to the colon. Histology of the proximal small intestine demonstrated impaired polarity and differentiation with blunted villi, having low expression of villin and lacking alkaline phosphatase. In addition, nearly all intestinal crypts were devoid of proliferative cells, suggesting loss of the transit-amplifying compartment. Ileal enteroids from *TTC7A* patients could not be maintained in culture (only several passages) compared to enteroids from healthy subjects. Furthermore, *TTC7A* mutant

enteroids demonstrated similar epithelial defects in polarity and proliferation, as did the intact tissue. Enteroids from patients with *TTC7A* mutations lack a central lumen and lateral membrane expression of tight junctional protein, ZO-1; they also lack proliferative cells. These studies provide further evidence that mutations in LGR5⁺ stem cells lead to altered intestinal epithelial phenotypes that mimic disease in vivo. Pharmacological studies using *TTC7A* mutant enteroids showed that inhibition of the RhoA/ROCK pathway could revert the disease-related phenotypes to normal, providing insight into how the previously uncharacterized protein, *TTC7A*, functions in normal intestinal epithelial polarization and differentiation (122). Broadly, these data contribute to a better understanding of the TTC protein family, as mutations in *TTC37* (i.e., trichohepatoenteric syndrome) are also shown to affect apical expression and localization of transport proteins in intestinal epithelial cells (123).

Cystic Fibrosis

Multiple lessons from studies of enteroids made from CF patients with differing mutations have already demonstrated their potential usefulness for understanding the pathophysiology of genetic diseases and directing the development of therapies. Using their innovative forskolin-induced swelling assay, Dekkers et al. (86) standardized a model from human and mouse enteroids that allowed comparison of rates of CFTR-dependent intestinal fluid secretion. This fluid secretion was lacking in patients with CF, but it could be partially restored with exposure of the enteroids to CFTR correctors and potentiators. Importantly, there were large differences in the efficacy of these drugs that were based on the CF mutation(s) present (86–88). Also, correction of the 508 CF mutations with CRISPR/Cas9 genome editing reestablished fluid secretion in the FIS assay, which could be reduced by CFTR inhibitors (124). Thus, studies with CF enteroids will likely be of continuing use in the evaluation of drugs designed to improve Cl secretion in the many CF mutations and the multiple ways in which they affect CFTR.

Colorectal Carcinoma

As one of the most important human cancers, colorectal carcinoma (CRC) is characterized for genetic and epigenetic contributions to the pathophysiology (125–128). Although sequencing analyses have revealed many of the major molecular pathways, mutated genes, and chromosomal translocations involved causing CRC, it is difficult to assess the functional contribution of each to both causation and to differences in drug response or patient outcome. Importantly, there are no easy-to-use methods to define how the genetic/epigenetic changes interact with environmental contributions, which almost certainly are some of the major inducers of CRC. Patient-derived intestinal enteroids offer the potential to be used as a model system to consider these interactions as well as to analyze the drug sensitivity of the tumor in a patient-specific manner.

One example describes the use of human enteroids in understanding genetic contributions to CRC; paired enteroids were made from adjacent healthy and tumor tissue from untreated CRC patients and used for DNA sequencing, RNA expression profiling, and drug screening (129). In this population, RNA expression analysis revealed upregulation of several cancer-associated genes, including *Prox1*, *BAMBI*, *Ptch1*, and *APCDD1* in the tumor enteroids.

High-throughput drug screening has begun using enteroids made from CRC to determine drug responses of individual cancers. Wnt secretion porcupine inhibitors, IWP2 or LGK974, reduced growth in a colonoid that carried a mutation in the negative feedback regulator RNF43. This suggests that inhibition of WNT secretion should be considered in trials for the treatment of a subset of CRC patients who carry the *RNF43* mutations. In another study, a novel orally available porcupine inhibitor that blocked secretion and activity of WNTs inhibited growth in R-spondin translocated CRC patient-derived xenografts (130). Both studies demonstrate the role of WNT signaling in CRC development and the potential utilization of WNT inhibition as a treatment option in a subset of CRC patients. Clustering the drugs based on their IC₅₀ values revealed a diverse range of sensitivities among the enteroids, but it importantly demonstrated that compounds with the same molecular targets had similar activity profiles (129). For example, the EGFR inhibitors cetuximab and gefitinib had a similar sensitivity profile on tumor enteroids. The effectiveness of several clinically used drugs was confirmed using the enteroid model system, including cetuximab in KRAS wild-type enteroids and Nutlin 3a in TP53 wild-type enteroids. However, there was a difference in the frequency of discordant mutations between the biopsy and organoids, suggesting the possibility of depletion or enrichment of a subclonal population in the tumor enteroid culture and acquisition of additional mutations during propagation. It was also noted that, in some instances, tumor enteroids had slower rates of proliferation compared to normal enteroids. This study (129) demonstrated that patient-derived enteroids could be used for high-throughput drug screening to test the efficacy of clinically available drugs and identify new therapeutic targets in a personalized or precision medicine approach. However, the caveat is that CRC involves continued mutations and changes in drug sensitivity over time in the tumors of individual patients. Enteroids made at one point in time are unlikely to reflect the continued evolution of cancer.

FUTURE DIRECTIONS

Normal Intestinal Physiology

Enteroids can be grown from each segment of the intestine ex vivo with no significant changes in genetics or physiology over time (89), and they recapitulate in vivo intestinal epithelial functions. By focusing on intestinal stem cells, enteroids can be used to study many aspects of normal intestinal physiology and pathophysiology. The organoids offer the opportunity to examine developmental biology, whereas enteroids more closely mimic adult intestine. As an example of a major gap in understanding normal intestinal physiology, the expression levels of different transporters along the human intestinal horizontal and vertical axes are currently unknown. Therefore, enteroids can be used to create an age-dependent human atlas of transporter expression along the horizontal and vertical axes of the human intestine.

Intestinal Pathophysiology

Enteroids can facilitate research into intestinal pathophysiological mechanisms by focusing on genetic diseases, cancer, gastrointestinal disorders, and host-pathogen interactions. The initial studies reviewed here suggest that intestinal stem cells are altered in multiple acquired GI disorders as well as in genetic disorders. However, the extent of the changes and which

aspects of diseases are preserved in the enteroids must be determined prospectively. This is true both for complex diseases such as IBD, in which there are multiple contributing components, and for simpler GI diseases, in which there are changes in the expression of specific genes or proteins. In spite of this concern, enteroids made from patients with several GI diseases have exhibited persistent changes in their genotypes and phenotypes. Adult stem cells in enteroid cultures have been shown to retain the gene expression profile of their donor, enabling the study of differences in gene expression profile, mutations, and some epigenetic changes. Enteroids derived from patients with some genetic diseases mimic the in vivo status of the disease and can be used to study the pathogenesis on a molecular and cellular level. In addition, studying gene expression profiles in enteroids is almost certain to enable the identification of novel mutations and lead to improved prenatal screening and early diagnosis of genetic diseases.

Regeneration

Enteroids can be used for regenerating damaged intestinal tissue. Upon transplantation of murine enteroids to damaged colon epithelium, engraftment and integration of the transplanted cells were observed (131). This raises the possibility of using human enteroids for regenerative medicine in cases with extensive mucosal damage, such as ulcerative colitis and postendoscopic mucosal resection. Interestingly, in chemical colitis models in mice, both small intestinal- and colonic-derived enteroids were able to increase the rapidity of colon ulcer healing, with the state of differentiation of the small intestinal enteroids determining whether the transplanted enteroids retained small intestinal or colonic characteristics.

Gene Therapy

Mutations in patient-derived enteroids can be corrected using gene therapy. For example, CRISPR/Cas9-mediated correction of CFTR mutations in enteroids derived from CF patients restored the function of CFTR and resulted in a functional FIS assay (124). This finding demonstrates that gene therapy is possible for adult stem cells and can potentially be used as a therapy to transplant “corrected” healthy intestinal stem cells for regeneration of the intestinal epithelium in patients with genetic diseases (such as microvillus inclusion disease). Because of still incomplete ethical and technical aspects of this use of enteroids, this area is in an early stage of development in the United States.

Enteroids can be grown in large amounts and even in a way that includes interaction with nerves. Such models are being considered for use in transplantation for short gut syndrome (132, 133). An advantage is that the patient can be used as the source from which to grow the enteroids, minimizing the risk of rejection and the need for long-term immunosuppression. However, although organoids have already been interacted with enteric nerves to demonstrate stimulated contractions (134), coordination requires pacemakers in the intestine. Dysmotility disorders are a major cause of GI morbidity with no currently available adequate way of improving symptoms. Thus, the use of the enteroids for increasing functional intestinal surface area is likely to reproduce the severity of the dysmotility disorders unless a way is found to establish normal pacemaker function and neural coordination of peristalsis in the remaining and added intestine.

Drug Screening/Biobanks

The majority of GI drug screening is performed on human cancer cell lines and animal intestines. These do not reflect characteristics of normal human intestine or the heterogeneity in the human population. The lack of studies in normal human intestine has probably contributed to the failure of ~90% of drugs undergoing development (135). Biobanks can be built using enteroids from different normal or genetically and clinically diverse individuals to enable the study of pharmacological compounds in a heterogeneous population. Consideration of the number of subjects needed to make such an approach meaningful for drug development will vary based on the type of drug.

Enteroids can be used for high-throughput screening assays to assess the efficacy and toxicity of compounds. Several recent examples include the screening of enteroids derived from patients with multiple forms of intestinal atresia, which resulted in the identification of Rho kinase inhibitors that could restore cell polarity (122). Also, drug screening using a small biobank of enteroids derived from patients with colorectal cancer revealed a diverse range of sensitivities among the enteroids and demonstrated that compounds with the same molecular targets had similar activity profiles (129).

Personalized or Precision Medicine

Enteroids have great potential for use in the patient-specific study of a disease and personalized approaches for drug development. Patient-derived enteroids can be used for high-throughput drug screening to determine the best drug combination for treatment. For example, drug screening in a biobank of 20 enteroid lines derived from CRC patients revealed that the growth of a single culture with *RNF43* mutation was affected by WNT secretion porcupine inhibitors, suggesting that inhibition of WNT secretion should be tested for the treatment of a subset of colorectal cancer patients (129).

Limitations

Enteroids are a model of the epithelial cells of the intestine, whereas organoids are slightly more complex, containing mesenchyme, some endothelial cells, and smooth muscle. However, neither is a complete intestine, as they lack neural innervation, immune cells, vasculature, and microbiome; enteroids also lack mesenchyme, including myofibroblasts. Additionally, the current models lack mechanical stress (peristalsis) and luminal and basolateral flow. However, each of these components can be added either alone or together, as is currently being done for some T cells (136) and macrophages (N. Zachos, unpublished data). The future of studies with human enteroids involves increasing the complexity of the model to create a “human intestine on a chip” that more closely resembles the intact human intestine. A final warning is that although great excitement exists in finally having a model of human intestine that gives access separately to the villus-like and crypt-like compartments, what will be learned to advance understanding of normal and abnormal intestinal function can be accessed only in the future.

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Figure 1.

(a) Human jejunal enteroid grown as a two-dimensional monolayer following five days of WNT removal to induce differentiation. Polarity is illustrated by apical expression of MUC2 and basolateral expression of E-cadherin; differentiation is indicated by the presence of MUC2, which is lacking in undifferentiated enteroids. Subpanels *i* and *ii* are the XY projections, and subpanel *iii* is the XZ projection. MUC2 is shown in green and E-cadherin in red. (b) Anion secretion occurs in both crypt-like undifferentiated jejunal enteroids but also in villus-like differentiated enteroids. The illustration shows active anion secretion (indicated by the short-circuit current response under voltage clamped conditions) induced

by forskolin (FSK), which is greater in undifferentiated enteroids but present (~20% of undifferentiated) in differentiated enteroids (JY Yin & CM Tse, JHU, unpublished data).

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Figure 2.

(a,b) Current textbook models of separate Na absorptive and Cl secretory cells. In the absorptive cells, NHE3 and DRA compose neutral NaCl absorption, which functions in parallel with Na- and H-linked amino acid apical uptake processes and Na-linked D-glucose uptake. In the Cl secretory cells, CFTR and CaCC (calcium-activated Cl channel, the molecular identity of which is not known) represent the apical Cl channels identified. HCO₃ exists either through CFTR or via apical DRA (not known), exchanging the secreted Cl for HCO₃. (c) Revised model of Na and Cl transporting cells based on studies of undifferentiated and differentiated three-dimensional enteroids. Transport proteins present in

both carry out NaCl absorption and anion secretion, with the major difference being an absence of NKCC1 in differentiated enteroids.

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