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Stem cell derived models: tools for studying role of microbiota in intestinal homeostasis and disease

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Abstract

Purpose of the review: In this review, we will summarize the recent progress made in generating stem cells-based organoid and enteroid models of the gastrointestinal tract and their importance in understanding the role of microbes in intestinal epithelial homeostasis and disease.

Recent finding: Intestinal stem-cell derived culture systems are self-organizing three-dimensional organotypic cultures that recapitulate many cellular, architectural and functional aspects of the human intestine. Progress has been made in the development of methods to incorporate additional cell lineages and physiological cues to better mimic the complexity of the intestine. Current model systems have facilitated both the study of gastrointestinal infections and interactions with normally non-pathogenic microbial residents of the GI tract. These studies have illustrated how live microbes, or their metabolites, ligands and virulence factors influence epithelial cell differentiation, maintenance, repair, function and intestine development.

Summary: Organotypic models are invaluable tools for studying host-microbe interactions that complement *in vivo* experimental model systems. These models have evolved in terms of complexity and fidelity. The stem-cell based models are already at forefront for studying host-microbe interactions and with continued development, the future looks even more promising.

Keywords

organoid; enteroid; host-microbe interaction; bacteria; epithelium

Introduction

The mucosal lining of the human gastrointestinal tract is an important site of direct contact between the host cells and microbes. These epithelial-microbe interactions play an important role in gut homeostasis. Multiple systems exist to study the molecular and physiologic interactions between the gut mucosa and resident microbes. Previously host-microbiota interactions were modeled with either *in vitro* transformed cell lines or *in vivo* animal

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Conflict of interest

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models. Models employing cell lines are characterized by a homogenous cellular population and limited relevance to the *in vivo* environment (1). Animal model systems can recapitulate the complexity of the human gastrointestinal tract with the caveat of species-specific differences in intestinal physiology, anatomy, diet and resident microbiota (2). Stem-cell-derived *in vitro* model systems can bridge the gap between these models. Stem cell-based intestinal models originating from either pluripotent or adult human stem cells recapitulate the cellular, architectural and functional aspects of the human intestine. Here we highlight recent advances in the use of microbiota-associated stem cell-based model systems to study gastrointestinal health and disease.

Stem cell derived model systems

Several versions of stem-cell derived organotypic models have been developed. Organoids are three dimensional structures derived from either human embryonic stem cells (ESC) or induced pluripotent stem cells (iPSCs) (Figure 1). Organoids have been generated for multiple gastrointestinal tissues including liver, pancreas, stomach, small intestine and colon. Directed differentiation of stem cells along particular gastrointestinal lineages is driven by specific growth factors (3, 4). However, to date only organoids resembling duodenum and colon have been developed using this approach. In general, organoids consist of an inner lumen enclosed by an organ specific heterocellular polarized epithelium and an outer mesenchymal layer. However lately attempts have been done to generate mesenchyme free small intestinal and colonic organoids (5*). This emergent model will open possibilities for investigating epithelial specific functions and interactions of the iPSC derived organoids. Moreover, the intestinal organoids resemble fetal epithelial cells with ability for further differentiation and maturation upon *in vivo* transplantation (6).

Enteroids, conversely, are derived from adult tissue-specific stem cells and maintain structural and functional similarity to the tissue of origin (Figure 1). Enteroids are composed only of a mature epithelial layer without mesenchyme (7). The epithelial only nature of enteroids facilitate formation of two-dimensional monolayers which are heterocellular and demonstrate epithelial polarity mimicking *in vivo* conditions (8). Enteroids maintain genetic diversity and epigenetic signatures of their cellular origin over time (9*, 10).

Both enteroids and organoids express all major cellular lineages of the intestinal epithelium including enterocytes, goblet, enteroendocrine, Paneth cells, and stem cells. Additionally, they can be chemically and genetically manipulated and can be expanded and cryopreserved indefinitely (4). The various organotypic models offer specific advantages and limitations (Table 1). The specific research question addressed will dictate the use of one model system over another.

Organotypic models to study host-epithelial interactions in the gut

For exploring the role of microbes in intestinal homeostasis, several different methods have been developed to expose microbes to organoids and enteroids (Figure 2). Live microorganisms or their products can be microinjected into the lumen of 3D organoids and enteroids. However, this is resource intensive, technically challenging and accumulation of

debris inside the lumen interferes with direct and uniform interaction between microbes and epithelium (11). Recently developed apical-out enteroids overcome this limitation by providing free access to the apical surface. This adaptation, however, is not useful for studying microbes that preferentially interact with the basolateral surface of the epithelium (12*). Another approach used for co-culturing microbes with organoids, is spontaneous infection of fragmented organoids with microbes followed by resealing of organoids into 3D structures (13). Several host-microbe co-culture studies have also used human intestinal enteroids (HIEs) in two dimensional forms. These monolayers when grown on cell culture inserts with permeable membrane, provide access to both apical and basolateral sides of the epithelial layer (14). With several possibilities available, the choice of microbial exposure method should be determined by the accessibility to the physiological route of exposure, stem cell model used and the availability of resources. Another refinement of monolayer culture involves establishing an air-liquid interface wherein apical surface of the monolayer is devoid of any media (15**). This approach was successfully used for co-culturing *Cryptosporidium* with murine intestinal enteroids and did not affect epithelial cell viability or function. This technological advancement will thus enable investigation of epithelium derived factors that affect microbial growth and colonization within the intestine without interference from media derived nutrients from apical side.

Studying host-microbe interactions *in vitro* is challenging because most microbes residing in the GI tract are anaerobic whereas epithelial cells require oxygen for viability. The lower oxygen concentration inside 3D organoids allows survival of anaerobic bacteria, however, it is not clear if it permits the growth of strict anaerobes (6, 16). Two separate research groups have developed simple, cost-effective methods to develop oxygen gradients across the epithelial monolayer and successfully co-cultured strict anaerobic gut bacteria with the oxygen requiring enteroid monolayers (17**, 18). With these innovations, stem-cell based models have overcome the limitations of conventional aerobic *in vitro* models in studying host-gut microbe interactions.

Attempts have also been made to incorporate additional physiologic conditions such as peristalsis and intestinal luminal flow into these models using bioengineering approaches. Microfluidic-based gut organoid flow chip devices have been developed for three-dimensional HIOs to maintain steady state flow conditions through the organoid. This device enables removal of debris from the organoid lumen with a simultaneous exchange of nutrients (19). Similarly, an intestine-on-chip device was developed for 2D models to simulates the mechanical and physiological features of the human intestine (20*, 21). This *in vitro* platform consists of differentiated enteroid monolayer as well as endothelial cells on the opposite sides of a porous membrane, an intact mucus layer, oxygen gradient and a mechanism capable of generating luminal flow and peristalsis. Furthermore, this device can sustain complex human stool microbiota for a number of days without compromising epithelial function (20*). This success in recapitulating complex cross talk between multiple tissue lineages and anaerobic bacteria will potentially provide new insights on the mechanisms involved in gut microbiota associated gastrointestinal homeostasis and disease.

Use of microbe incorporated stem-cell derived models for studying gastrointestinal homeostasis and diseases

The technological advances in organotypic models have led to multiple studies that investigate the role of gastrointestinal microbes in epithelial maintenance, repair, regeneration, and barrier function. Here we highlight some of these studies that focus on gastrointestinal homeostasis and disease.

Epithelium regeneration, repair and maintenance

Intestinal stem cells (ISCs) play an important role in epithelium homeostasis by balancing between self-renewal and epithelial differentiation, regeneration and maintenance. ISCs were considered more protected from pathogens because of their deep location within the intestinal crypts. A recent study using murine and human enteroids provided the first direct evidence that the toxins produced by *C. difficile* can damage colonic stem cells. Colonic tissue from mice infected with toxigenic *C.difficile* had decreased expression of stem cell markers and were less efficient at generating enteroids, suggesting an effect on stem cells. This was associated with the production of the toxin TcdB by *C. difficile*. The study was further able to demonstrate that the stem cell damage delays epithelial repair and regeneration (22**). Organoids are also being explored to understand the protective effect of non-pathogenic bacteria on ISCs (23, 24*). A recent work unraveled the mechanism by which resident gut microbiota protects ISCs and promotes epithelial regeneration after oxidative stress (23). Muramyl dipeptide (MDP), a common bacterial motif recognized by the NOD pattern recognition receptor, reduced cell death due to apoptosis in enteroids following irradiation. Furthermore, an increase in NOD2 expression, expansion of Lgr5+ stem cells and decreased ROS generation were observed in MDP-stimulated irradiated enteroids. Another study explored how gut microbes restores homeostasis in the setting of inflammation. The authors stimulated murine enteroids with *Lactobacillus reuteri* and observed that under physiological conditions, *L. reuteri* maintained proliferation of ISCs by activating Wnt-β catenin signaling pathway (24*). Under pathological conditions caused by inflammatory mediator TNF, *L.reuteri* was able to reverse the epithelial damage by maintaining the number of ISCs and stimulating epithelial proliferation. Together these studies show that the protective or pathologic effect of individual microbial species on ISCs can be well investigated using stem-cell based microbial co-culture models and may provide clue to targets that can promote epithelial regeneration following injury, infection or cancer therapy.

Epithelial lineage differentiation and function

Recent studies have used organotypic models to show the effect of microbes on the differentiation of individual cellular lineages of the intestinal epithelium (25–27). Using *Clostridium ramosum*, Mandic *et al* demonstrated the role of gut microbes in programming differentiation of colonic ISCs towards enterochromaffin (EC) lineage of the epithelium (28**). This effect of *C. ramosum* on murine ISCs was indirect since a bacterial lysate stimulated release of serotonin from enteroids. Increased serotonin in turn programed differentiation of stem cells towards serotonin secreting EC cells by changing the expression

of genes involved in maintaining a balance between secretory and absorptive lineages of the intestine. Organotypic models have been employed to investigate the role played by different cellular lineages in microbial recognition and stimulating their growth within the gut. It has been demonstrated that sparse cellular lineages in human intestinal organoids/enteroids can be enriched (29–31). Leveraging on this property of stem-cell models, tuft cells were expanded in mouse intestinal enteroids using IL-13. The increased population of tuft cells helped in investigating the response of tuft cell towards parasitic helminth *Trichinella spiralis* infection (32). In conjunction with animal work, enteroids were used in this research to identify the key components such as parasite receptors, intracellular transduction proteins and other molecules involved in the signaling cascade induced in tuft cells after parasite infection. The activation of signaling cascade resulted in release of IL-25 cytokine by the enteroids indicating activation of type 2 immunity. Similarly, the role of M cells in uptake of *Shigella flexneri* was confirmed by inducing expression of M cells in enteroids (31, 33*). The study showed a significant increase in intracellular bacteria in enteroids with increased M cells. The enhanced accessibility to these rare cellular populations via stem cell models demonstrates they are an ideal platform for understanding the role played by the scarce intestinal cell types in recognizing microbes and mediating reciprocal responses (32, 33*).

Intestinal development

Alterations in gut microbiota structure and function impacts intestinal health and physiology in newborns and infants. The use of stem cell based models have shown an association between early life microbiota and intestinal maturation (34, 35*). Abo *et al*; used enteroids generated from conventional, germ-free and co-housed mice to demonstrate that the microbial exposure in early life positively influences epithelial regeneration, proliferation and repair of ISCs by controlling expression of erythroid differentiation regulator-1 in ISCs (36). Another group introduced human neonatal microbiota into mouse enteroids to understand the impact of early life microbiota on intestine development. In the study, microbiota from preterm infants, as opposed to microbiota from term neonates, induced epithelial proliferation marked by increased expression of stem cell gene markers and formation of large budding enteroids (35*). Although it is still under debate if microbial colonization occurs before or at birth; it is important to know how initial colonization of the gut affects intestinal development and function. Organoids, due to their resemblance to immature fetal epithelium can also be used to address these questions, thus providing a leverage in investigating the role of microbes in early life intestinal development. In fact, an earlier study found that introduction of *E.coli*, an early gut colonizer, into the immature organoids resulted in epithelial maturation as indicated by increased differentiation of enterocytes, mucus production and improved epithelial barrier (6). The microbe-stem cell-based co-culture models therefore may prove instrumental in advancing our understanding of the role played by microbes in intestinal development, physiology and function during postnatal adaptation.

Modeling gastrointestinal infections and diseases

Organotypic models have served as reliable model system for studying the pathophysiology of various infectious agents involved in gastrointestinal diseases including bacteria, viruses and parasites. This has been comprehensively reviewed previously (37–42*). Traditional *in*

vitro and *in vivo* models of GI tract were lacking in their ability to study certain infections either because of lack of pathogen infectivity or poor disease manifestations (1, 2). It is beyond the scope of this review to provide details of each pathogen individually; we have compiled a list of key steps in the pathogenesis of several gastrointestinal infections that have been recently studied using these models (Table 2). Receptors for the recently emerged coronavirus, SARS-CoV2, were found on apical side of human small intestinal and colon derived enteroids permitting the study of gut infection by this virus (43, 44). It was also shown that the virus primarily infects and replicates in mature enterocytes and to a lesser extent in undifferentiated enteroids. Moreover, the infected epithelium elicited expression of cytokines and genes associated with interferon I and III responses (45**). The rapidity with which organotypic models can be used to study a new viral pathogen modeling demonstrates the power and flexibility of these systems.

With the ability to generate enteroids from diseased gut epithelium and successful use of CRISPR/CAS technology in creating organoids from modified pluripotent stem cells (46), stem cell based models have been used to study mechanistic aspects of the role of microbes in various multifactorial diseases of the gut including celiac disease (9*), Barrett's esophagus (47), inflammatory bowel diseases (48), necrotizing enterocolitis (49) and cancer (50**). For example, repeated exposures to genotoxic *E. coli* over multiple passages of human pediatric enteroids induced unique mutational signatures in the enteroid epithelium (50**). Interestingly, similar mutational signatures were found in colorectal cancer patients providing a direct evidence for the role played by microbes in colorectal cancer development or progression. Although still in its infancy to accurately mimic such complex diseases, human enteroids have the potential to address the questions relevant to long term effect of presence or absence of common gut residents on individual human health and disease.

Conclusions

Role of microbes in host health and disease is well established but the mechanistic details are not well understood. Organotypic models offer an invaluable tool for studying host-microbe interactions and have filled the gap between other *in vitro* and *in vivo* experimental model systems. Till date, most studies have concentrated on studying individual microbial strains specifically pathogens and limited attempts have been made to co-culture the complex microbial community of the human gastrointestinal tract with the organoids or enteroids. Moving forward, for a clear understanding of the host-microbe interactions of the gut it will be necessary to include complex microbiota into these models. Significant new advances are made in these models with the introduction of other physiological and cellular components of the intestine (51, 52). These components play a critical role in modulating both microbes and epithelium. Thus, it will be important to incorporate them in microbe-stem cell-based co-culture models as well. Nonetheless, organotypic models are already at forefront for studying host-microbe interactions and with the continuous evolvement, the potential of these models to recreate complex host-microbiota interactions of the GI tract looks promising.

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Key points

- Intestinal organoids and enteroids recapitulate various structural and functional features of human intestinal epithelium.
- Intestinal organoids or enteroids have served as valuable models for several studies focused on understanding the effect of microbes on intestinal homeostasis and disease.
- Incorporation of more than one microbial species in these models should be considered to create more physiologic relevance.
- Current strategies used for developing stem cell based engineered models of the intestine need further improvement to more precisely recapitulate in vivo conditions of the GI tract.
- These model systems offer exciting opportunity for understanding the complex interactions between the intestine and its microbiota.

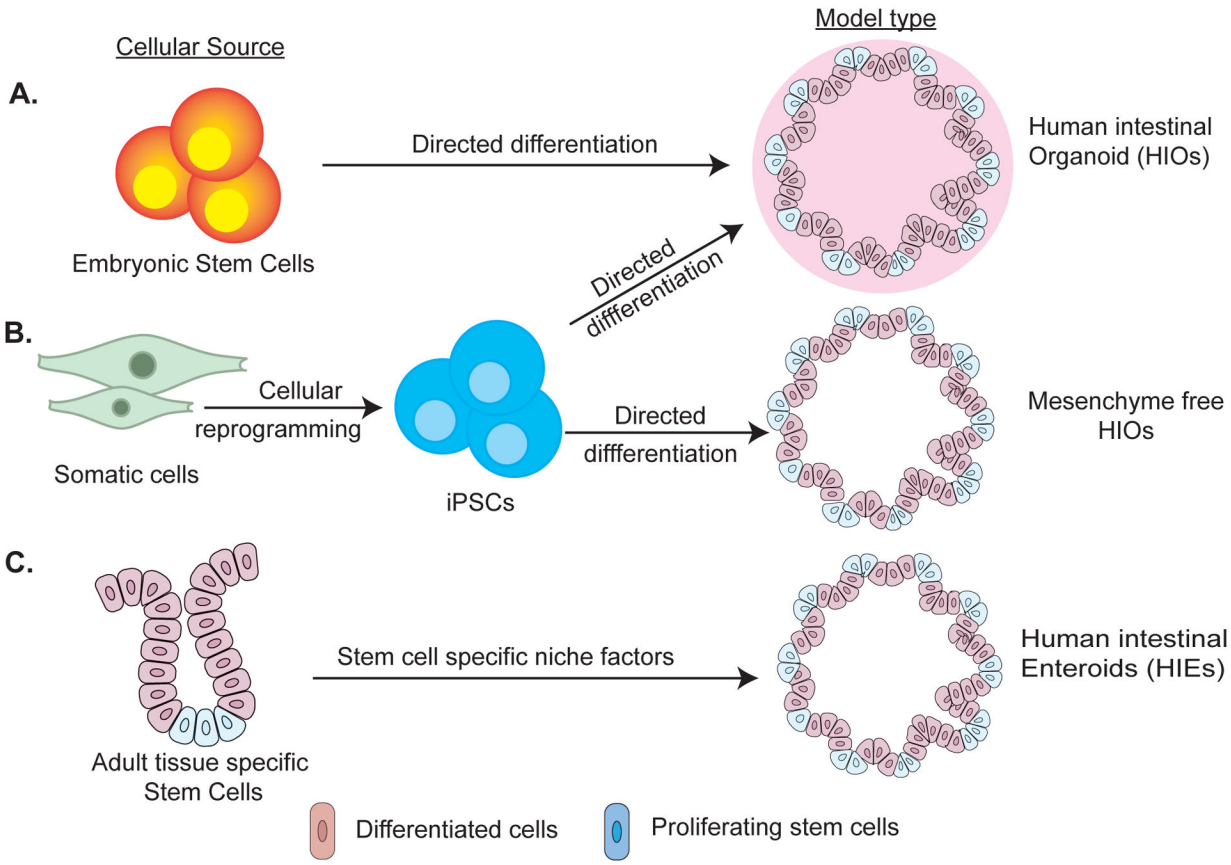

Figure 1:

Schematic illustration summarizing generation of human intestinal stem-cell derived models from A) embryonic stem cells (ESCs) B) induced pluripotent stem cells and (iPSCs) C) adult stem cells. Both ESC and iPSC are differentiated into definitive endoderm followed by generation of CDX2 expressing spheroids. Spheroids are grown in 3D matrix to generate HIOs with mesenchyme. Mesenchyme free HIOs are generated from iPSCs by inhibiting BMP/TGF-β signaling pathway followed by sorting for gut progenitor cells that are differentiated into small or large intestine in specific growth media. HIEs are generated from stem cells present in the biopsies or tissue sections obtained from region of interest followed by their growth in 3D matrix in undifferentiated or differentiated state using specific growth factors.

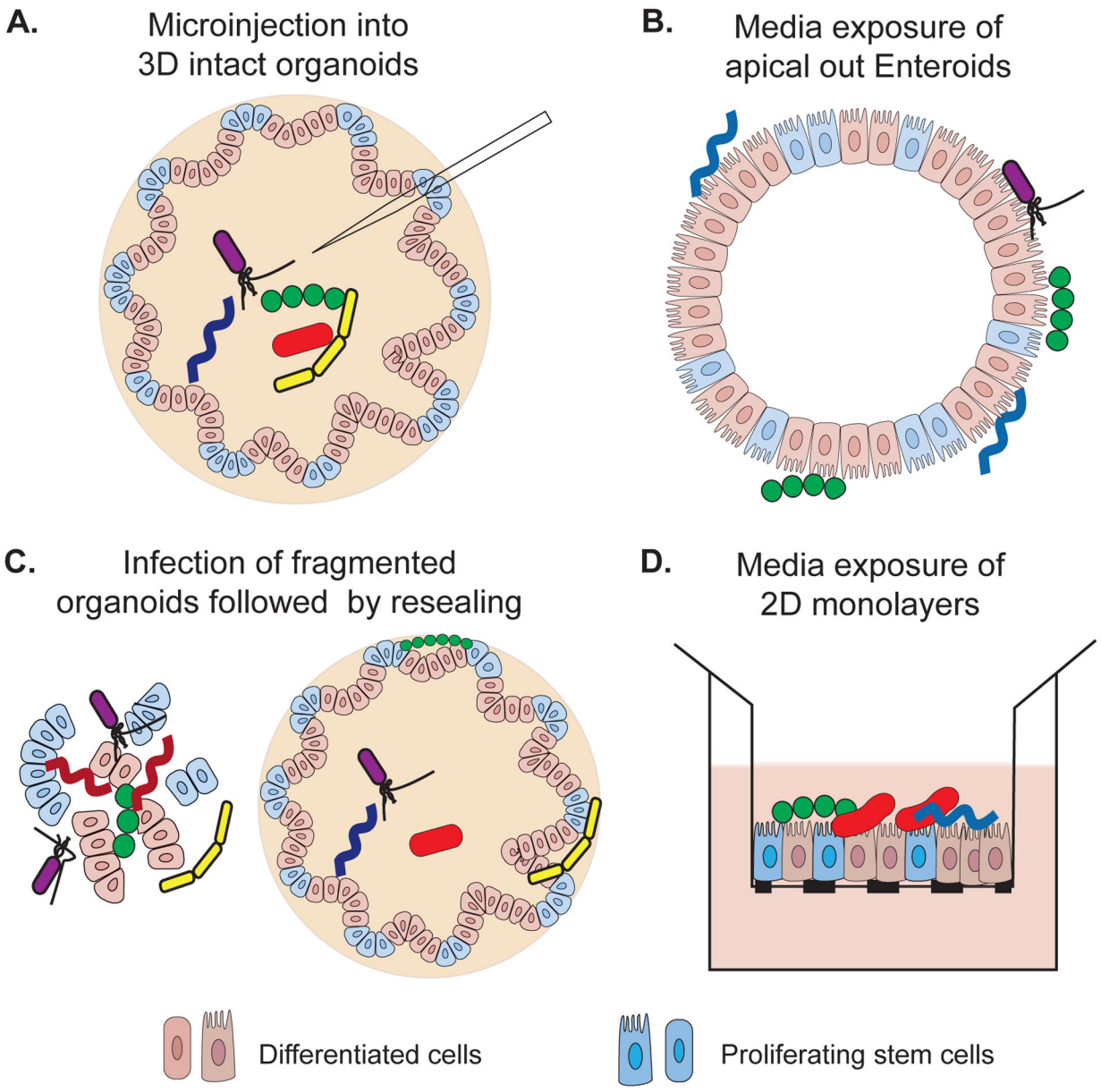

Figure 2:

Methods used for exposing human intestinal stem-cell derived models with gut microbes. A) Microinjection is used for introducing microbes into the lumen of 3D organotypic models enabling microbe-epithelium interaction at the apical side. B) Polarity of enteroids in apical out HIEs is reversed by removing surrounding matrix and microbes are added in the outer media providing apical access. C) Dissociated 3D organotypic cultures are mixed with microbial species of interest. Microbes may get incorporated into the lumen or left outside in the matrix of reformed 3D structures. D) Organotypic models can be enzymatically dissociated to form 2D polarized monolayers and microbes can be added either on the apical or basolateral side.

Table 1:

Key features of human intestinal stem-cell derived models

Organotypic Model	Cellular Components	Key Features	Limitations
iPSC and ESC derived Organoids	Epithelium and mesenchyme	• Resemble immature duodenum or colon • Self-organizing and renewing • Can be genetically manipulated	• Limited regional specificity • Bidirectional epithelial-mesenchymal signaling may interfere mechanistic studies
iPSC derived Mesenchyme free Organoids	Epithelium	• Resemble small intestine and colon	• Not yet completely characterized
Adult stem cell derived Enteroids	Epithelium	• Retain structural, functional and genetic features of cellular source • Amenable to 2D form	• Ethical concerns hinder tissue availability • No human model with underlying mesenchyme • Fragile and small for microinjection
Adult stem cell derived inside out Enteroids	Epithelium	• Easy access to apical side • Three dimensional	• No access to basolateral surface • Loss of enclosed lumen

Table 2:

Main pathogenesis steps recently studied using stem-cell derived models

Aspects of intestinal infections studied	Pathogen studied	Main findings	Reference
Host susceptibility	Human astrovirus	Pathogen replication rates in human enteroids can be donor dependent	(53, 54)
	Shigella flexineri		
Pathogen receptors involved in epithelial adherence	*Salmonella enterica* serovar *Typhi*	Serovar specific role of phospholipid transporter (YrbE) in epithelial adherence	(55, 56)
	Enteroaggregative *E. coli*	Aggregative adherence fimbriae II were involved in adherence and pathogenesis to human enteroids	
Preferential route of pathogen entry	*S. Typhimurium*	Preferentially invades apical surface	(12*, 33*)
	Shigella flexineri, Listeria monocytogenes	Attaches to basolateral surface	
Cells involved in pathogen replication	Human astrovirus SARS-CoV-2	Astrovirus shows tropism for multiple cell types whereas SARS-CoV-2 was mainly found in enterocytes	(44, 53)
Epithelial response	Human norovirus	RNA-seq analysis showed activation of JAK-STAT pathway	(57)
Effect on epithelial barrier	*C. difficile*	Effect on epithelial barrier function is strain and toxin specific	(58, 59)
	Enterovirus	Not all enteric viruses affect epithelial barrier integrity	
Activation of innate immune response	Enterovirus71	Induction of type III interferon immune response upon infection	(54, 58)
	Shigella flexineri	Release of proinflammatory cytokines and chemokines by HIE	
Treatment strategies	*Shigella flexneri*	A *S. flexneri* targeting phage prevented its adhesion and invasion to HIEs	(60*, 61)
	C.difficile	Bacitricin inhibits translocation of toxin B into the cytosol of epithelial cells thereby preventing their disruption	