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Title
Intestinal Organoids

Permalink
https://escholarship.org/uc/item/9nv2728m

Journal
Journal of Pediatric Gastroenterology and Nutrition, 64(2)

ISSN
0277-2116

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Publication Date
2017-02-01

DOI
10.1097/mpg.0000000000001411

Peer reviewed

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J Pediatr Gastroenterol Nutr. Author manuscript; available in PMC 2018 February 01.

Published in final edited form as:

J Pediatr Gastroenterol Nutr. 2017 February ; 64(2): 180–185. doi:10.1097/MPG.0000000000001411.

Intestinal Organoids: New frontiers in the study of intestinal disease and physiology

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Abstract

The development of sustainable intestinal organoid cell culture has emerged as a new modality for the study of intestinal function and cellular processes. Organoid culture is providing a new test-bed for therapeutic research and development. Intestinal organoids, self-renewing three-dimensional structures comprised of intestinal stem cells and their differentiated epithelial progeny allow for more facile and robust exploration of cellular activity, cell organization and structure, genetic manipulation, and vastly more physiologic modeling of intestinal response to stimuli as compared to traditional two dimensional cell line cultures. Intestinal organoids are impacting a wide variety of research into gastrointestinal pathology. The purpose of this review is to discuss the current state-of-the-art and future impact of research using enteroids and colonoids (organoids grown from the small and large intestines respectively).

Keywords

Enteroid; Colonoid; *lgr5*; intestinal stem cell; Mini-gut; intestinal organoid; ex vivo; gut; precision medicine

Introduction

Accurate models of biological systems are vital to the advancement of all biosciences. The pursuit of a reliable and accurate *ex vivo* model of intestinal function has attracted much interest, owing to the limitations of existing two dimensional (2D) immortalized cell line-based systems. Heretofore, intestinal epithelial cells (IECs) would rapidly undergo anoikis (a form of apoptosis) following isolation, preventing establishment of primary, non-transformed IEC cultures. Building upon the earlier success of short term intestinal crypt cell culture on collagen coated vessels, the Clevers laboratory succeeded in the creation of the first self-renewing, non-transformed mini-gut organoid culture[1]. This significant advancement created a new way to study the function of the intestinal epithelium, yielding

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Conflicts of Interest

There are no conflicts of interest.

Author Contributions

T. Wallach performed the literature review, synopsis, and authored the manuscript

J. Bayrer assisted with literature search, writing, and editing of the manuscript.

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an accessible platform for basic and translational experimentation in a physiologically relevant context.

The pursuit of an ex vivo model of intestinal function has been a long one, beginning with initial success in growing adult crypt cells on collagen coated vessels, which could be propagated for short periods of time[2]. This evolved through efforts by Ootani and others to establish a culture system based on an air-liquid interface in which neonatal intestinal mucosa (epithelium and mesenchyme) formed long lasting organoid structures.[3] The common ground between these systems is the requirement for mesenchymal fibroblasts, without which propagation is impossible. In 2009, Sato et al established a platform for organoid growth and propagation that broke free of mesenchymal dependence, instead employing a defined set of intestinal stem cell niche factors. This work simplified the process of growing and maintaining intestinal crypt cultures and expanded the variety of potential source material.

An organoid is defined as a miniature organ grown *in vitro.* It can be produced via adult multipotent stem cells or induced pluripotent cells (iPS) cultured in a stromal replacement such as Matrigel. Importantly, adult intestinal multipotent stem cells are limited in differentiation to intestinal epithelium whereas iPS cells, derived from embryonic stem (ES) cells or reprogrammed from adult tissue, can be driven to differentiate into a broader array of cells.[4] When referring to intestinal organoids, the source of the harvested tissue further defines the structure and characteristics of the resulting organoid. Tissue harvested from the small intestine will recapitulate small intestinal function and structure, and is termed an “enteroid,” based on NIH-sponsored consensus guidelines[5]. Similarly, cells harvested from the colon will recapitulate the colon, creating a “colonoid.” Intestinal organoids can be produced from either isolated intestinal stem cells or stem cell-containing intestinal crypts. Both methods result in growth of a three dimensional structure of epithelial cells. The structure of these cells will resemble their *in vivo* organization when supplied with appropriate exogenous growth factors and basement membrane scaffolding (*e.g.* Matrigel). All intestinal cell types are represented in this structure, including *lgr5*$^{+}$ crypt-based columnar stem cells (CBC ISC, hereafter simply ISC), quiescent stem cells (QSCs), transit-amplifying (TA) cells, absorptive enterocytes, goblet cells, enteroendocrine cells, and Paneth cells. ISCs are defined as cells that regenerate crypt-villus units for a lifetime, and are possessed of long-term self-renewal and multipotent differentiation. They can differentiate into all intestinal cell types. ISCs, marked by the Wnt-amplifyng gene *lgr5*, retain the capacity to functionally expand and recreate the crypt-villus structure. QSCs represent the reserve stable of ISCs activated following *lgr5* CBC loss[6]. The TA cells rapidly amplify to create tissue mass and precursors for differentiation. Each of the other listed type is a fully differentiated cell, with absorptive enterocytes serving the engine of nutritional absorption, goblet cells creating a protective mucus layer, EECs secreting gastrointestinal hormones, and Paneth cells producing protective antimicrobial peptides[7]. The ability to recreate this structure allows researchers to create a stable, modifiable *ex vivo* model of intestinal tissue function. The self-renewing property of intestinal organoids enables indefinite propagation and expansion using standard cell culture techniques, allowing an increase in research throughput as compared to prior techniques of intestinal stem cell culture that necessitated continually sourced starting material. Intestinal stem cells can be harvested from either

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animal or human subjects, including both surgical and endoscopic biopsy specimens. iPS cells (can also form intestinal organoids[8]. In this review we will focus on enteroids and colonoids derived from ISCs (both human and murine).

Enteroids and colonoids have wide ranging applications and potential uses. The ability to characterize intestinal epithelial development is substantial, with the establishment of the crypt/villus axis enabling cell tracking and fate analysis[1]. Many of the uses of organoids for fate analysis and developmental biology purposes are well covered in recent reviews, including recent publications by Zachos *et al*[9] and Carulli *et al*[10]. In addition, newer techniques where organoids are linearlized into epithelial sheets are allowing for development of new high throughput screens with more physiologically accurate epithelial structure, which may supplant immortalized cells in usage for high-throughput analysis and permeability analysis. For the purposes of this review, we will focus on the current state of the field with regard to clinical and translational applications of enteroid and colonoid technology.

Organoid Growth and Maintenance

Enteroids were initially created from small intestinal crypts containing *lgr5*$^+$ ISCs[7] harvested from mice[1]. Harvested crypts were induced to self-renew and differentiate by the addition of Epithelial Growth Factor (EGF), R-Spondin, and Noggin to standard growth media[1]. When cultured in basement membrane extract with these growth factors, ISCs will self-renew, differentiate, and self-organize into enteroids, with the lumen on the inside of a 3-dimensional structure (Fig 1). These assemblages resemble the macroscopic structure of the intestines with polarized epithelial cells forming a simple columnar epithelium with distinct crypt and villus domains. As it stands, the interior-oriented apical organizational structure presents a challenge to existing experimental techniques, as we will discuss below.

The components of the growth media have been well studied and optimized for sustained *ex vivo* growth. The basement membrane extract is produced by Engelbreth-Holm-Swarm (EHS) tumor cell line and mimics the native supportive stroma[11]. This allows for the ISCs to attach to a superstructure, supporting epithelial cell survival through integrin signaling, and suppressing anoikis. R-spondin, a secreted protein mainly expressed by subepithelial fibroblasts, binds to the *lgr5* receptor, suppressing degradation of Wnt receptors and potentiating Wnt activation, a key ingredient for the maintenance of the ISCs[12,13]. Noggin is a secreted glycoprotein and BMP antagonist, and has proved essential to maintenance of enteroid cultures. Without Noggin, enteroids lose *lgr5* expression and cease proliferation after 2 weeks[1]. BMP is a mesenchymal product thought to be responsible for driving intestinal differentiation, but may also regulate ISCs[14]. EGF is required for epithelial cell survival and long term culture[15,16].

Human enteroids can be created using ISCs harvested from intestinal tissue in much the same manner as murine enteroids. Human enteroids require the addition of the canonical WNT ligand Wnt-3A, a p38 inhibitor (small molecule SB202190) and TGF-β inhibitor (typically ALK 4/5/7 inhibitors like A83-01 or SB431542)[17]. Human cells require addition of exogenous Wnt to grow, likely secondary to insufficient endogenous Wnt production

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when compared to murine organoids[14]. Addition of p38 inhibitors is necessary to suppress secretory lineage differentiation, which if allowed to proceed unchecked would deplete the supply of ISCs necessary to maintain the culture. It is not clear at this time why this is a requirement for human and not murine culture. TGF-β inhibition is necessary for human cell culture likely due to increased sensitivity to the BMP/ TGF-β pathway, in which TGF-β will inhibit WNT-driven cell proliferation[14]. Colonoids are at this point more challenging to maintain in culture. While colonoids have been successfully cultured using methodology similar to enteroids[14], they have been found to have a different response to Wnt-3A signaling than enteroids, and may be more successfully cultured using Wnt-3A, prostaglandin E2, and nicotinamide in addition to standard mouse small intestinal culture media[18].

Enteroids and the Study of Gastrointestinal Infectious Diseases

Enteroid and colonoid technology creates a novel way of modeling enteric infectious processes, enabling both the interrogation of host-pathogen interactions and the search for therapeutic targets. This is an issue of tremendous importance, as diarrheal disease remains one of the world's largest health issues. Pioneering research demonstrates the superiority of the intestinal organoid system over immortalized cell culture for study of the molecular pathogenesis of these diseases.

Rotavirus remains a significant cause of morbidity and mortality despite the increasing prevalence of oral vaccines, with a pediatric mortality of over 450,000 children under 5 annually[19]. Enteroid culture allows a much closer and more accurate modeling of the infectious process than previously possible by supporting the full viral life cycle[20]. Furthermore, intestinal organoids have been shown to be more permissive for infection than immortalized lines[20], again more closely modeling the in vivo infectious process. Although at this time enteroid culture has primarily complemented previous work done using 2D immortalized lines, the fidelity of the pathologic model increases confidence in recent therapeutic intervention studies demonstrating the effectiveness of interferon-α and ribavirin inhibition of viral reproduction[20].

Organoids have also been used to study host-bacterial pathologies, including many of the most common and problematic infectious agents. *Clostridium difficile* remains the main antibiotic-associated cause of diarrhea in the United States, with an estimated healthcare cost of over 1 billion dollars annually[21]. The pathology of *C. difficile* has been studied extensively in immortalized cell lines, but the development of organoids has allowed for confirmation of previous findings and further advances into the cellular mechanisms of cytotoxicity. Investigators have used human colonoids to examine the pathologic role of C. difficile toxins on intestinal epithelium[22,23]. Previously it was known that in immortalized cell lines, *C. difficile* toxin A and B function by binding uncharacterized host receptors that trigger the inhibition of Rho GTPases, leading to disorganization of the cytoskeleton, loss of tight junctions, and disruption of signaling and the cell cycle[24]. In addition, Toxin B is known to diminish expression of the NHE3 sodium/hydrogen exchanger[25], resulting in increased diarrhea. Researchers confirmed and extended these findings using colonoids exposed to *C. difficile* via microinjection of cultured bacteria, fecal material from *C. difficile*

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infected patients, or purified *C. difficile* toxins TcdA and TcdB[22]. These works confirmed TcdA-mediated epithelial tight junction damage and identified NHE3 upregulation as a target for therapeutic agents aimed at interrupting the process by which *C. difficile* creates a hospitable environment for itself. It also provides evidentiary support for the improvement generated by lactobacilli, which upregulates NHE3 expression[22]. Greater understanding of the pathogenesis and mechanisms by which *C. difficile* causes illness provides new possible targets for therapeutic intervention and primary prevention.

Other studies have demonstrated the utility of live bacterial co-culture with intestinal organoids. Zhang and colleagues studied the mechanism and time course of salmonella infection of enteroids[26]. Following only a brief incubation period, they found significant bacterial adherence and invasion of the intestinal epithelial cells. RNA expression and immunofluorescence analysis identified a decrease in the important tight junction proteins ZO-1, Occludin, and Claudins, thereby demonstrating a direct impact on epithelial tight junctions. Salmonella infection further elicited the onset of the inflammatory cascade, with NF-$_K$B activation and an expansion in expression of inflammatory cytokines from the enteroid culture. The ability of enteroids to recapitulate the infectious process in *ex vivo* culture presents a valuable opportunity to develop therapeutic agents that go beyond simple bactericidal mechanisms and directly block the translocation and infection of the host. Enteroids and colonoids are a significant improvement in reductive modeling over traditional epithelial cultures, by providing the native crypt structure, epithelial cell subtypes, and intestinal epithelial tight junctions that resemble those found in vivo.

The exploration of the intestinal organoid culture as a model for infectious pathology presents a tremendous opportunity for further discovery and intervention in infectious enteric diseases. Currently, 9 out of 10 drugs fail in clinical trials, many due to differences between animal models and human biology[27]. The ability to recreate human biology *in vitro* vastly increases the applicability of pharmacologic research, both broadening the approachable targets and validating initial efficacy in a more robust model. While significant work has been done using immortalized cell lines to delineate the basics of infectious pathophysiology for many agents, the enhanced fidelity of enteroids and colonoids, as well as the differences noted in protein expression (possibly due to interactions between epithelial cell types) illustrate the value of these models. The main limitation of this model is that only intestinal epithelial cells are represented, therefore potentially important contributions from immune or mesenchymal cells may be missed.

Enteroids and Diarrheal Disease

Diarrheal disease remains one of the greatest causes of morbidity and mortality in the world, accounting for approximately 4% of all deaths worldwide, and 1.2 million pediatric deaths annually[28]. Research into the mechanisms of pathogenic insult and the onset of secretory diarrhea has benefited from the emergence of enteroids. In all diarrheal illness there is a reduction in sodium absorption primarily due to inhibition of the brush border NHE Na^+/H^+ exchanger potentiated by interference with the $Cl^-/HC0_3^-$ exchanger DRA[29]. In enterotoxigenic diarrhea, activation of CFTR contributes significantly to anion secretion[30]. Using genetic and pharmacologic approaches, researchers have confirmed that enteroids

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express these key transporters at physiological levels and maintain their ion exchange function[31]. Given these findings, enteroids are well-suited for study of intestinal anion/fluid homeostasis. Specifically, enteroids and colonoids can be used to explore the precise mechanisms involved in pathologic alterations contributing to diarrhea.

Initial work in the area has identified a novel therapeutic target for severe secretory diarrheas such as *Vibrio cholera* infection. Using human enteroids, Foulke-Abel *et al* were able to identify that NBCe1, a basolaterally located cAMP mediated bicarbonate transporter, and CFTR were integral to secretory diarrhea pathways whereas other bicarbonate transporters including NKCC1, a basolateral sodium-potassium-chloride transporter, are not. Using a high throughput forskolin induced swelling assay in conjunction with inhibitors of NKCC1, Foulke-Abel and colleagues demonstrated pharmacologic blockade of NKCC1 only partially impacts swelling. This suggests residual stimulated fluid secretion is supported by other loaders. Indeed, pre-treatment with an NBCe1 inhibitor generated intracellular acidification in enteroids following forskolin stimulation. This observation suggested an inability of cells to replace bicarbonate secreted by CFTR and therefore treatments interfering with NBCe1 function could diminish movement of bicarbonate in intestinal cells, preventing both fluid loss and resultant acidemia[31].

Enteroids and the Study of Cystic Fibrosis

One of the initial benefactors of the advent of intestinal organoids has been research in CF intestinal disease. From better delineating the pathogenesis of CF intestinal complications (constipation, cellular hyperproliferation, dysbiosis, inflammation, cancer risk), to investigating possible treatment options, the research platform provided by both *ex vivo* culture of mouse CFTR knockout enteroids and enteroids derived from CF patients has wide implications in the field.

CFTR is a major pathway of Cl^- and HCO_3^- ion efflux from intestinal epithelial cells. Knockout of CFTR in mice creates a physiologically relevant alkaline shift[32]. Enteroid technology has allowed better understanding of the impact of a CFTR mutation on pH balance[33], specifically showing CFTR non-function creates an alkaline shift in the intestinal epithelial cells through local cell-mediated processes. The impact of this effect is substantial, as an alkaline pH is known to encourage cellular hyperproliferation by favoring cell cycle progression[34,35] which in turn sets the stage for the development of neoplasia[36]. Given that CF patients have a 6 fold increased risk for intestinal cancer and that research shows the potential to decrease this epithelial alkalosis by reducing Cl^- concentration in the epithelium[33], this presents an interesting opportunity to attempt to modify the risk of CF-related intestinal neoplasia.

Perhaps even more revelatory for CF research is the opportunity to use enteroids as models for drug development and genetic therapy. Intestinal colonoids have already been adapted for high-throughput screening of CF therapeutic agents using a forskolin model. Exposure to forskolin[37] increases intracellular cAMP, activating CFTR. In non-CF cells, this leads to increased fluid secretion into the lumen. A successful high-throughput screen using a microfluidics capture system based on optical measurement of colonoid swelling in response

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to forskolin exposure[38] has been designed. In this assay, colonoid size is monitored by an automated imaging system after exposure to an osmotic challenge in the form of forskolin and a potentially protective therapeutic agent. An agent that successfully augments mutant CFTR function displays similar enlargement to wild type colonoids. This relatively simple assay may allow for improvements in personalized therapy (given the capacity to grow colonoids from a specific patient), as well as a sizeable impact on drug development.

With the recent emergence of CRISPR, the ability to culture enteroids and colonoids offers a model of human cellular response to genomic alterations, especially given the ability to stably passage multiple generations and monitor the persistence and effects of the engineered changes over time. Schwank *et al* have shown that the CRISPR/Cas9 system can correct F508 mutations in organoids harvested from CF patients, with full rescue of the CFTR phenotype[39]. While targeted stem cell therapy may not be applicable for CF patients given the multi-organ nature of the disease, the research potential and implications for the field more broadly is substantial.

Enteroids, Colonoids, and Oncology

Antitumor pharmacologic research has long benefited from traditional cell culture of immortalized cancer cell lines, but these cultures have significant limitations. Namely, lack of personalization to an individual's tumor, introduction of genomic instability by immortalization, and inability of the cell lines to match the function and structure of the original heterogeneous tumor. Intestinal organoid technology offers the capability of growing cultured tumors specific to an individual, allowing for rapid improvement in personalized medicine through the use of tumor-tailored therapeutics.

Proof-of-principle for this concept was recently published by the Clevers laboratory. Tumor organoids (tumoroids) were successfully cultured from colorectal cancer alongside colonoids from normal tissue[40]. Genetic fidelity between the original tumors and the tumoroids proved to be consistent. Furthermore, Clevers was able to adapt the tumoroids to a roboticized high-throughput drug screen to determine anti-neoplastic drug sensitivities. New work is underway to expand this methodology into xenograft assays, in which tumoroids are implanted into mice allowing for more broad experimentation with anti-neoplastics in the context of an animal host[41]. In addition, other work into using colonoids as a model of response to radiotherapy has been validated[42], potentially furthering the role of truly individualized oncologic treatment for intestinal cancers.

Short Gut, Intestinal Regrowth, and Intestinal Organoids

Intestinal organoids represent a possible therapeutic avenue for the treatment of short gut, with multiple lines of research into using organoids to either repair damaged intestines *in situ* or to potentially tissue engineer intestines for what essentially amounts to auto-transplantation.

In 2012, Yui *et al* demonstrated intestinal organoids can seed mouse colonic mucosa damaged via the dextran sulfate sodium (DSS) colitis model and repair the epithelial wound[43]. Murine colonoids were instilled into the damaged colons via enema. One week

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following instillation, colonic lesions showed significant recovery with organoid engraftment, and by 4 weeks the treated colons demonstrated repaired and healthy colonic structures with appropriate epithelial barrier function. Later studies confirmed these findings, and interestingly, found that the transplanted *lgr5+* stem cells maintain the identity of the bowel region they were derived from, allowing the creation of heterotopic rests with different functional capabilities[44] and suggesting tissue-specific programing occurs during intestinal stem cell production. The potential of this therapy to repair intestinal inflammatory damage is broad-reaching, most notably in therapy for inflammatory bowel disease. While recognizing the limitations of adapting processes successful in murine models to human therapy, with further characterization of signaling the ability to implant functioning heterotopic rests suggests potential therapeutic options for patients with partial intestinal resection, specifically to provide functions of lost intestine which cannot be restored by adaptation, such as ileal-dependent absorption of bile salts and vitamin B12.

Work into *in vitro* growing of functional intestinal grafts continues, with organoids serving as the basis for several successful studies into tissue engineered intestines. Multiple groups[45,46,47] have shown the ability to use organoids to grow both colon and small intestinal structures by seeding a polyglycolic/poly-L-lactic acid (PGA/PLLA) scaffold with ISCs, and growing them *in vivo* via implantation into immune-deficient mice. This results in a tubular structure which functionally reproduces the architectural, absorptive, and barrier membrane aspects of the intestinal lumen. While artificially grown intestinal segments lack glia and neurons necessary for motility, these initial efforts yielding epithelial structures grown on an artificial superstructure are a promising step towards the development of true tissue-engineered intestines. The capacity for auto-transplantation of the small and large intestine has real and near-term implications for pure epithelial disorders such as microvillus inclusion disease or tufting enteropathy.

Conclusion and Limitations

Enteroids have already enabled new avenues of research into intestinal function and pathology, but there is still significant room to improve in modeling the true physiology of intestinal function. The enteroid is solely an epithelial structure, and even in terms of modeling intestinal epithelial responses it is limited by our current inability to fully form signaling gradients such as BMP found in human tissue. The structural limitations of enteroids create a challenge in exposing the apical aspect to experimentation, as well as in techniques for imaging or functional endpoints. In addition, they fail to capture the input and interactions of other cell and tissue types key to understanding a variety of intestinal pathology such as inflammatory or motility diseases. However, new developments in the field promise to help alleviate some of these shortcomings.

One of the main limitations of the current model of enteroid culture is mechanical, specifically that the three dimensional structure of enteroids can limit certain types of experimentation, as the apical aspect is inside the enteroid. Techniques such as microinjection have circumvented this issue for initial studies, but in order to access other applications, new techniques are required, specifically in monolayer culture of enteroids and colonoids. In a recent publication, Scott et al were able to successfully demonstrate a

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protocol for growing intestinal stem cells in proliferative and confluent monolayers, based on matrix of type 1 collagen and laminin isotypes[48]. Other investigators have worked on similar methods of 2D enteroid culture, with varying degrees of success. This alleviates some of the structural restrictions in using 3D enteroid culture, providing easier methodology for exposure of the apical surface, imaging of the cells, and lending itself more to high throughput screens. There is even the potential to supplant CaCo-2 as the barrier membrane of choice for pharmaceutical investigations, allowing for a more physiologic understanding of oral drug delivery.

Progress is being made in improving the capability of organoid culture to fully mimic *in vivo* responses by adding layers of complexity to the reductionist model. Recent successes in co-culture of murine enteroids and murine intraepithelial lymphocytes (IELs) suggest the potential for even more robust modeling of infectious pathophysiology, inflammatory disease, and intestinal healing[49]. The ability to further expand the capacity of this ground breaking *ex vivo* model may provide the tools to explore not only the activities and interactions of intestinal cells, but the broader superstructure surrounding it.

In the past five years, the development of *in vitro* modeling of organ function has changed the face of biological research. Far from the approximations of intestinal epithelial cell interactions possible with immortalized cell lines, this new technology has already allowed for a fuller understanding of host-pathogen interaction in the intestines, increased understanding of congenital diseases from cystic fibrosis to microvillus inclusion disease[50], and a introduced a whole new field of potential therapeutic intervention for short gut or surgically altered intestines. It has provided a major new platform for personalized medicine, with individualized tumor organoid culture only the first wave of applications for the testing of personalized therapy under development, especially in light of the NIH Precision Medicine Initiative. As additional work proceeds in co-culture with other cell types, and our ability to model *in vivo* systems in an *in vitro* capacity improves, the potential of this technology is enormous.

Acknowledgments

Sources of funding:

K12HD07222

T32DK007762

F32CA163092

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What is Known

- Intestinal Stem Cells (ISCs) are self-renewing pluripotent stem cells inhabiting the intestinal crypt
- ISCs can be experimentally induced to create a functional recapitulation of the large and small intestinal epithelium

What is New

- Intestinal organoids are currently being used to study a variety of host-pathogen interactions
- Intestinal organoids serve as a useful platform for drug screening and personalized medicine
- Disease specific *ex vivo* models can be created from patient-derived material.
- Intestinal organoids may someday support the creation of tissue-engineered small intestines

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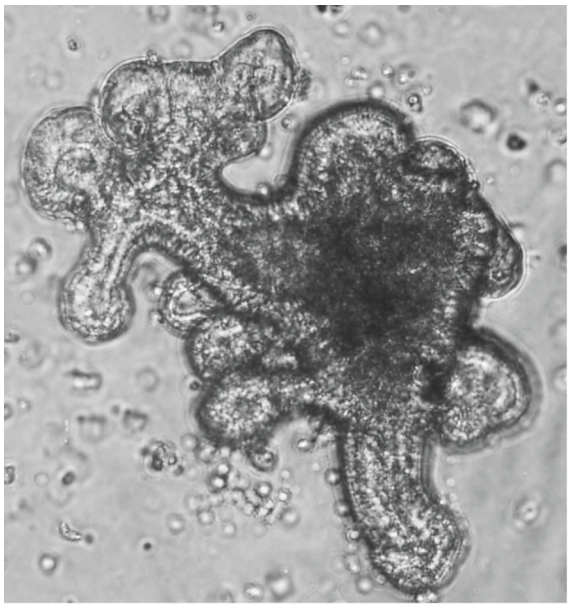

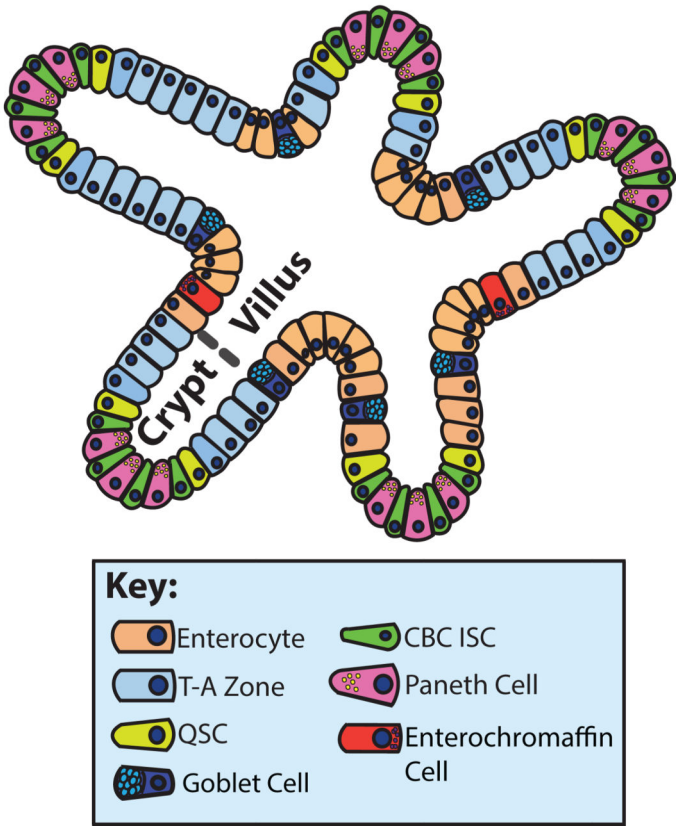

Figure 1.

A) Murine small intestinal organoid. Note the distinct crypt and villus domains **B)** Illustration of typical intestinal organoid structure and distribution of intestinal epithelial cell types