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Intestinal stem cell-on-chip to study the human gut

Authors

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Abstract

The gut is a tubular organ responsible for digestion and harbors our intestinal microbiome. This organ is composed of a multitude of specialized cell types arranged in complex barrier-forming crypts and villi covered by a mucosal layer controlling nutrient passage and protecting from invading pathogens. The development and self-renewal of the intestinal epithelium is guided by niche signals controlling the differentiation of specific cell types along the crypt-villus axis in the epithelium. The emergence of microphysiological systems, or organ-on-chips, has paved the way to study the intestinal epithelium within a dynamic and controlled environment. In this review, we describe the use of organ-on-chip technology to control and guide these differentiation processes *in vitro*. We further discuss current applications and forthcoming strategies to investigate the mechanistic processes of intestinal stem cell differentiation, tissue formation, and the interaction of the intestine with the microbiota in the context of gastrointestinal diseases.

Keywords

Pluripotent stem cells, intestinal stem cells, organ-on-chip, microbiota, gastrointestinal disease

Introduction

The gastrointestinal tract is a central organ system that enables the ingestion, digestion, absorption, and the utilization of processed nutrients to fuel the overall body. Within this system, several organs are responsible for digestion and the uptake of nutrients, including the stomach, the small intestine, and the large intestine. The gut forms a tubular structure whose central lumen is lined with a protective mucosa composed of a monostratified epithelium forming invaginations (or crypts) and finger-like protrusions (or villi). This epithelium extends to the apical side of the gut, allowing nutrient uptake and acting as a first line of protection against invading pathogens (Otani & Coopersmith, 2019). A crypt-villus structure is seen in the small intestine, while only crypts are present within the colon. Both the initial development and continuous self-renewal of the intestinal epithelium are guided by niche signals in addition to cell-autonomous processes that initiate the outgrowth and differentiation of intestinal stem cells (ISCs), driving proliferation and differentiation following a crypt-to-villus axis in the small intestine. This process gives rise to progenitor cell lineages showing absorptive or secretory characteristics that subsequently terminally differentiate into specific cell types within the epithelium, including enterocytes, enteroendocrine cells, Paneth cells, goblet cells, Tuft cells, Microfold cells (M cells), and cup cells (H. Clevers, 2013). A rich tissular environment surrounds the epithelium and includes smooth muscle cell layers, the enteric nervous system, and connective tissue that contains arteries and lymphatics and is abundant in fibroblasts and mast cells (Mowat, 2003).

The last few decades in biomedical research have led to a number of robust experimental strategies and techniques to study intestinal physiology. Yet, *in vitro* studies aiming to model the gut mucosa have mostly been limited to static monocultures and co-cultures. In contrast to these older models, recent developments that use microphysiological systems allow scientists to probe both the intestinal epithelium and its environment in a dynamic and physiologically relevant manner (Mosig, 2017).

In this review, we will emphasize the transitions from immortalized cell lines to stem cells and describe their use in combination with gut-on-chip models. These “on-chip” models are defined as three-dimensional (3D) cell cultures arranged as multiple cell layers actively perfused by microfluidics for medium exchange to enable an improved tissue formation with increased lifespan. We will highlight few applications of these gut-on-chip models to understand gastrointestinal diseases and forthcoming strategies to investigate human gut physiology.

Cell sources for gut-on-chip models: from immortalized cell lines to stem cells

Immortalized cell lines have been used extensively to study the intestine. For example, the colorectal adenocarcinoma cell line Caco-2 had been originally used to study the intestinal epithelial barrier as these cells differentiate spontaneously into a monolayer of enterocytes when reaching confluence (Sun, Chow, Liu, Du, & Pang, 2008). However, the use of cell lines with a cancerogenous background is associated with important limitations, including their spontaneous dedifferentiation and the lack of cellular diversity. Further, immortalized cell lines such as Caco-2 are not able to reflect a patient-specific genetic background to enable a personalized approach to study individual mechanisms of disease-related conditions. To overcome these limitations, the use of human pluripotent stem cells (hiPSCs) or adult stem cells has been explored as a promising alternative to established cell lines. Human adult ISCs maintain an organ-specific imprint and epithelial maturity and possess a patient-specific background, thus enabling personalized studies (Kasendra et al., 2018; Sontheimer-Phelps et al., 2020). Biopsy-derived cells and intestinal organoid models have emerged as powerful tools to recreate tissue complexity and high cellular diversity *in vitro* (Beumer & Clevers, 2021; H. Clevers, 2016). ISCs are a valuable cell source to grow organoids with organotypic self-patterning, thereby recreating essential microanatomical features of the gut (H. C. Clevers, 2019). However, the availability of primary tissue for the isolation of ISCs is limited and requires a rapid processing of *ex vivo* samples. Hence, human embryonic stem cells (hESCs) and hiPSCs are interesting alternatives as they can be used to generate intestinal cells through directed differentiation (Spence et al., 2011). The use of hiPSCs allows for the generation of multiple differentiated cell types from a patient-specific background with an unlimited supply of human stem cells stored in biobanks (Huang et al., 2019). Furthermore, hiPSCs from patients with a genetic background of interest can be obtained by reprogramming from a multitude of easily accessible primary cell sources, including urine, blood, and skin (Takahashi & Yamanaka, 2016). Nevertheless, a significant drawback to the use of pluripotent stem cells can be found in the difficulty to produce tissues that reach full maturity. The recapitulation of whole embryonic development can be challenging due to a lack of knowledge of all the required biochemical cues driving the differentiation and morphogenic processes. One of the most novel *in vitro* approaches in intestinal mucosa modeling is the use of microfluidically perfused organ-on-chip seeded with cell lines, primary cells, or stem cells (Jalili-Firoozinezhad et al., 2019; Kasendra et al., 2018; Maurer et al., 2019; Sontheimer-Phelps et al., 2020; Tan et al., 2018; Tovaglieri et al., 2019). A clear benefit of these types of models is that they allow for better control of the microenvironment. One example includes the addition of flow on Caco-2 cells to differentiate them and express the typical cell markers of essential cell types of the small intestine (Goblet- and Paneth-, enterochromaffin-, and brush border-like cells) (H. J. Kim & Ingber, 2013). Further, flow conditions stimulated an increased expression of mucin-2 with the

development of a thickened mucus layer covering the epithelial tissue, indicating the functional relevance of goblet-like cells (Kasendra et al., 2018; Shin & Kim, 2018). Perfused organ-on-chip further facilitates a physiological cellular crosstalk by aiding in the outgrowth of organotypic microstructures such as villi and crypts (Kasendra et al., 2018; Maurer et al., 2019). In addition, these epithelial cell layers have been combined in a number of studies with endothelial cells and immune cells using various tissue engineering approaches (Huh et al., 2013; Kasendra et al., 2018; Maurer et al., 2019). These studies highlight the potential of organ-on-chip as a tool to manipulate the microenvironment by facilitating the long-term growth and co-differentiation of different cell types to recreate some typical microanatomical features of the human intestine. However, given the cancerous background of the Caco-2 cell line data generated with these models, the data should be interpreted with caution as this cell line has several limitations in its differentiation potential to individual intestinal cell types compared to adult stem cells or induced pluripotent stem cells (Richmond & Breault, 2018).

Control and guidance of stem cell development on-chip

ISC-derived organoids have been used in recent years to experimentally address and dissect the individual spatiotemporal effects of different biochemical gradients on the growth and differentiation of ISCs. However, given the spherical nature of organoids, the stable recreation of physiological conditions over a longer period of culture has remained challenging (Wang, Kim, Hinman, et al., 2018). Microfluidically perfused organ-on-chip provides a platform to mimic *in vivo*-like biochemical cues to guide stem cell differentiation with typical microanatomical features such as villi and crypts (Figure 1).

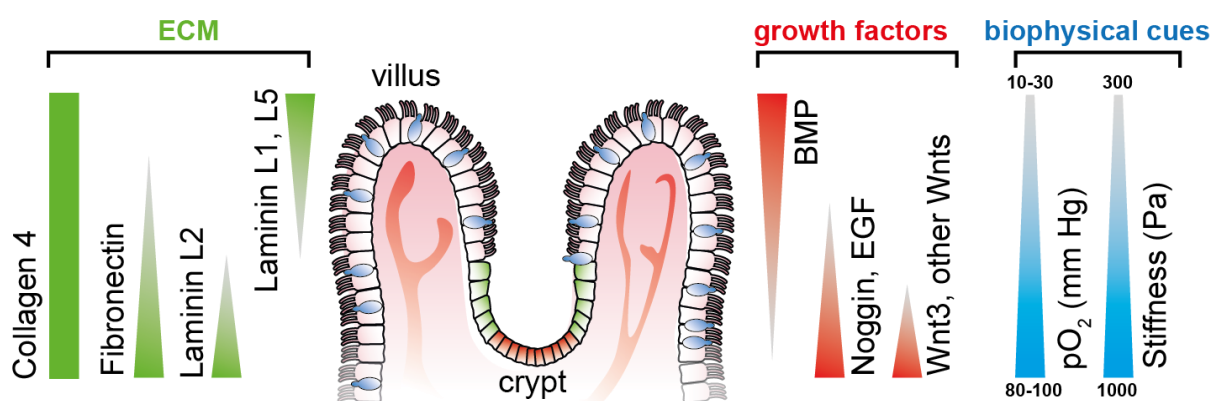


Figure 1. Defined gradients of biochemical and biophysical cues span along the crypt-villus axis in the small intestine, thereby determining cellular stemness and differentiation toward the tips of the villi. Triangles indicate only the gradient direction as the quantitative amounts

of growth factors and ECM protein are still to be elucidated. Illustration adapted from (Wang, Kim, Hinman, et al., 2018).

It has been long known that many aspects of cell fate are dependent on the geometry and mechanics of their microenvironment, including the induction of apoptosis (C. S. Chen, Mrksich, Huang, Whitesides, & Ingber, 1997), cellular differentiation (Dupont et al., 2011; Engler, Sen, Sweeney, & Discher, 2006), and proliferation rate (Murphy, McDevitt, & Engler, 2014). The presence of a flow through media stream has been demonstrated to be a requirement for the formation of 3D intestinal structures in cell line and primary cell-based intestinal models by modulating the biochemical availability of growth factors. The flow-dependent basolateral removal of the Wnt antagonist Dickkopf-1 and the elevated expression of the Frizzled-9 receptor were found to be required in the formation of epithelium villi-like protrusions (Shin, Hinojosa, Ingber, & Kim, 2019). Our existing knowledge on the individual growth factors driving ISC differentiation provides an excellent basis from which to engineer more realistic organ models with better accessibility. In this review, we are focusing on the use of the following two principal types of stem cells to differentiate and form intestinal tissue: adult stem cells and induced pluripotent stem cells. Both cell sources possess unique advantages and disadvantages in recapitulating the intestinal tissue (Table 1).

Table 1. Pros and cons of the use of adult stem cells and induced pluripotent stem cells for the generation of intestinal tissue models

Cell Source	Advantages	Disadvantages
Adult Stem Cells (ASCs)	<ul style="list-style-type: none"> - semi-autonomous multipotent differentiation capacity (J. Kim, Koo, & Knoblich, 2020) - preservation of tissue- and region-specific characteristics 	<ul style="list-style-type: none"> - could not be differentiated from non-epithelial tissue (endothelial cells, immune cells, etc.) - limited cellular diversity of ASC-derived organoids - limitations for expansion to large scale - limited by accessibility of tissue, difficult to isolate and purify from tissue
Induced Pluripotent Stem Cells (iPSCs)	<ul style="list-style-type: none"> - differentiation guided by external factors - somatic cells for reprogramming are easily accessible 	<ul style="list-style-type: none"> - variations in differentiation efficacy with limitations in cellular maturity - heterogeneity in generation of tissue-specific cell types

	<ul style="list-style-type: none"> - pluripotent and useable for generation of isogenic models with high cellular diversity - lower frequency of DNA mutations during <i>in vitro</i> culture compared to ASCs (Kuijk et al., 2020), preservation of genetic identity in vitro over a longer period of time 	
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A striking example of the potential of defined growth factor gradients to guide stem cell growth to intestinal tissues has been given by Wang et al. in the Transwell system (Wang et al., 2017; Wang, Kim, Gunasekara, et al., 2018). Arrays of artificial collagen-based crypts and villi structures were created by microfabrication and molding to recreate the topography of the intestine and the colon (Wang et al., 2017; Wang, Kim, Gunasekara, et al., 2018). The scaffold has been used to generate gradients of Wnt-3A, R-spondin, noggin, and gamma secretase inhibitor. These gradients enabled the establishment and maintenance of a crypt-villus axis composed of adult human ISCs that formed an epithelium containing proliferating progenitor cell compartments. Kasendra et al. recently presented a more complex model of the human duodenum that combines the advantages of organoids and organ-on-chip technologies (Kasendra et al., 2020; Kasendra et al., 2018). In this model, the tissue forms a polarized cell architecture under flow conditions and shows improved intestinal barrier function and differentiation of specialized cell subpopulations. This duodenum-on-chip system could exert mechanical stimulation to organoid-derived epithelial cells, resulting in improved tissue architecture. The perfusion of the tissue significantly improved cell-cell junctions and microvilli density, further improving intestinal barrier function. Remarkably, it was demonstrated that organoid-derived cells in the duodenum chip have an increased similarity in their global gene expression profile to human adult duodenal tissue compared to conventional static organoid culture. Further, in the duodenum-on-chip system, organoid-derived cells show an improved metabolic capacity compared to Caco-2 cells due to an increased induction capability and expression of CYP3A4 (Kasendra et al., 2020). The ability to integrate and analyze patient-specific stem cells from different donors within the chip represents a decisive advantage over the use of immortalized cell lines such as Caco-2 or HT-29. Stem cells not only allow for the reflection of the host's genetic background but also possess the ability to self-organize into an *in vivo*-like tissue structure with improved functionality. Both aspects are major assets for studies on drug uptake and drug-drug interaction and its metabolism to predict drug efficacy and

safety. Recently, Nikolaev et al. used synthetic hydrogels to guide the growth of adult human ISCs with intrinsic self-organization properties to tubular structures forming crypt- and villus-like domains (Nikolaev et al., 2020). Furthermore, in contrast to conventional organoids, the stem cell-derived tissue cultured in the hydrogel possesses a high regenerative potential, as demonstrated after stimulation with cytotoxic dextran sodium sulfate (DSS) to model colitis. The perfusion of the system was used to provide a continuous supply of nutrition as well as the removal of waste products and dead cells, allowing for a significantly extended lifespan of the organoid model with tenfold higher cell mass and the emerge of rare cell types such as M cells previously not observed in conventional organoid cultures (Nikolaev et al., 2020).

Similar approaches have also been demonstrated using hiPSCs. Workman et al. used epithelial cells isolated from hiPSC-derived human intestinal organoids and seeded them in a microfluidic perfused gut-on-chip. Flow conditions enabled the outgrowth of a 3D tissue model with spontaneous differentiation into epithelial-specific types, such as Paneth cells, goblet cells, enterocytes, enteroendocrine cells, transit amplifying cells, and Lgr5+ cells similar to *in vivo* cell populations (Workman et al., 2018). The immune responsiveness of the intestinal tissue after $\text{INF}\gamma$ stimulation was validated by the measurement of STAT1 phosphorylation after one hour and the upregulation of $\text{INF}\gamma$ -related genes three days after stimulation. Further, combined treatment with $\text{INF}\gamma$ and TNF resulted in an increased permeability of the epithelial cell layer. In another study, the same chip platform was used for the culture of stem cell-derived epithelial cells isolated from human colon organoids (Sontheimer-Phelps et al., 2020). Likewise, the OrganoPlate system, a plate-based cell culture system that allows pumpless media movement via a rocking platform, was used for cultivating hiPSC on a gel matrix to form a monolayered epithelial tube (Naumovska et al., 2020). The cells expressed the Paneth cell marker Lysozyme, the enterocyte cell marker Villin-1, and the neuroendocrine cell marker Chromogranin A. However, the expression of these cell marker proteins was diffuse and distributed over the entire tissue. Another shortcoming of the model consists in the absence of organized 3D epithelial structures.

These gut-on-chip systems strikingly demonstrate the huge potential of organ-on-chip platforms to precisely control environmental growth conditions, to guide the differentiation of stem cells, and to form improved intrinsically self-organized tissue with superior properties compared to traditional organoid cultures. Hence, the applications of ISCs include the possibility to investigate intestinal developmental biology and barrier function but also studies related to bacterial colonization, drug discovery, regenerative medicine, and responses to infections in a personalized approach (Crespo et al., 2017; Leslie et al., 2015; Negoro et al., 2018; Reiner, Sapir, & Parichha, 2020). The generation and integration of

hiPSC-derived immune cells will probably aid in the development of isogenic immune competent intestinal models derived from a single hiPSC line to avoid potential allogenic reactions.

Gut-on-chip models to study microbiome-host interaction

As a result of coevolution, niches of the human body are inhabited by a variety of commensal, mutualistic, and pathogenic microorganisms, including archae, bacteria, fungi, phages, protists, and viruses (Campion et al., 1988; Kapitan, Niemiec, Steimle, Frick, & Jacobsen, 2019; Ley et al., 2008; Lim et al., 2015; Lloyd-Price et al., 2017; Parfrey, Walters, & Knight, 2011; Qin et al., 2010). The gut provides a variety of niches serving as habitats for different ecological communities. On a macroscopic scale, biophysiological gradients determine the growth niche of microorganisms along the gut from the small intestine toward the rectum with an increase in the pH and the abundance of bile acids, whereas the availability of oxygen decreases. A change in the environmental conditions thereby determines microbial colonization with 10^4 CFU/ml bacteria in the small intestine up to 10^8 CFU/ml microorganisms in the terminal ileum, where elongated passage times and associated enrichment in nutrients provide ideal bacterial growth conditions (Donaldson, Lee, & Mazmanian, 2016; Kastl, Terry, Wu, & Albenberg, 2020). With that, the number of facultative anaerobic and obligate anaerobic species increases from the small intestine to the colon (Hayashi, Takahashi, Nishi, Sakamoto, & Benno, 2005; Thadepalli, Lou, Bach, Matsui, & Mandal, 1979).

The reliable monitoring of the microenvironment and its change upon tissue formation or bacterial colonization is key to control and understand the stem development and differentiation as well as the interaction of the intestinal tissue with the microbiota. Luminescent-based sensors integrated in organ-on-chip systems have been used to quantify medium dissolved oxygen levels (Rennert et al., 2015; Shah et al., 2016), cellular glucose consumption (Lin, Cherng-Wen, Roy, & Trau, 2009), and the changes of the pH by the release of lactate by metabolic active tissues or microorganisms (Kieninger, Weltin, Flamm, & Urban, 2018; Mousavi Shaegh et al., 2016; Weltin et al., 2017). To enable the growth of anaerobic bacteria in gut-on-chip, several platforms have been tailored to allow the stable formation of neighboring cell layers perfused with normoxic and hypoxic media streams. The characterization and quantification of microbiota-associated changes in the metabolic profile requires a reliable and continuous measurement of key metabolites and mediators of microbial colonization. Electrochemical enzyme-based biosensors and microbead-based microfluidic assays expand the ability of organ-on-chip systems to perform a detailed

monitoring of metabolic parameters including the formation of glutamine and glutamate (Backer et al., 2013; Tolosa, Wassum, Maidment, & Monbouquette, 2013), the release of reactive oxygen species (Enomoto, Matharu, & Revzin, 2013), and the secretion of various cytokines (Cui et al., 2018; Usuba et al., 2016). The determination of transepithelial electrical resistance (TEER) is another widely used method to assess barrier function and has been adapted in recent years for organ-on-chip platforms. In particular, the combination of impedance spectroscopy and electrical simulation has been used to quantify cell layer capacitance as a measure of villi differentiation in gut-on-chip models, which could be applied across different gut-on-chip platforms (van der Helm et al., 2019).

Gut-on-chip models have been used in recent years to better understand the complex interactions between the microbiota and their host. These systems allow to balance between robust and predictable approaches and the recreation of physiological-relevant culture conditions. The incremental nature of these models makes it relatively simple for them to be designed as “simple as possible and complex as required,” and they therefore offer additional flexibility in identifying contributing factors of host-microbiota interaction. The controlled escalation of biological complexity on the host side as well as in the composition of microbiota-derived factors and live microorganism communities will enable the elaboration and the proof of a complex interaction mechanism in a well-controlled and standardized environment provided by organ-on-chip platforms (Figure 2).

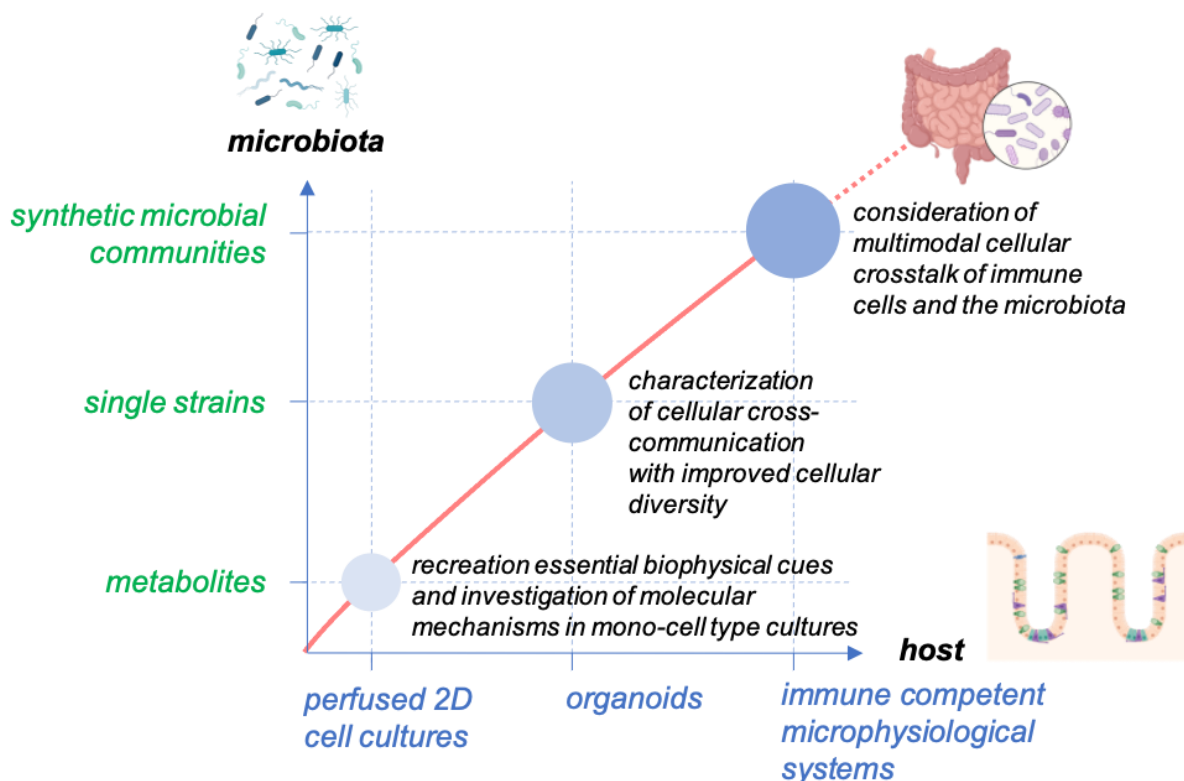


Figure 2. *Concept for studying host-microbiome interaction in gut-on-chip. The models enable the balancing of the cellular complexity of host tissue and provide the technical basis to facilitate the outgrowth of host cells to self-organized organoid structures with high cellular diversity. The systems allow the scaling of the biological complexity of the host and the microbiota to study the interaction of the host tissue with the microbiota from microbial metabolites to single microbial strains and diverse microbial communities.*

Examples of a successful application of this approach have already been provided. Sontheimer-Phelps et al. recently reported a system lined with primary patient-derived colonic epithelial cells (Sontheimer-Phelps et al., 2020). A mucus bilayer is built up in the chip by perfused epithelial cells and provides a suitable substrate for colonization with living microbiota to study host-microbiome interactions in the colon in more detail. Similarly, Shin et al. generated an anoxic-oxic interface on a chip inhabited with CaCo 2-cells and implemented *Bifidobacterium adolescentis* (Shin, Wu, et al., 2019). The system is able to co-cultivate bacteria for up to one week without the impairment of epithelial performance. This represents an interesting platform to assess mechanistic questions regarding Bifidobacteria-host interactions in the neonate period in more detail. Similarly, Zhang et al. reported the co-culture of the obligate anaerobic bacterium *Faecalibacterium prausnitzii* with colon organoid-derived epithelial cells in their physiomimetic platform for up to four days. In the study, the authors were able to uncover the mechanisms of an anti-inflammatory host response related to butyrate release with the downregulation of the gene expression of histone deacetylase complexes, Toll-like receptors, and nuclear factor kB (NF-kB) (Zhang et al., 2021). Different approaches have also been used to reconstitute the microbiome and its function *in vitro* in a more complex and detailed manner. A top-down approach consists of using the full microbiome derived from human stool samples, which would in principle mimic the *in vivo* situation ideally. However, it remains challenging to characterize its full composition in a short period of time before transfer into an *in vitro* model even under hypoxic conditions. Jalili-Firoozinezhad and co-workers managed to implement complex microbiomes in their *in vivo*-like system but pointed out that overgrowth and species shift due to artificial culture conditions might be problematic. Thus, a sophisticated pre-culture step (i.e., in large SHIME bioreactors) is still required to expand a defined microbiota prior to colonization experiments (Jalili-Firoozinezhad et al., 2019). However, these studies illustrate nicely the importance of advanced *in vitro* models offering the ability to precisely dissect individual microbiota in humans.

Although conventional organoid cultures already provide invaluable insight into detailed ISC dynamics, their application for long-term studies or the dissection of host-microbiota

interaction has some limitations. The long-term exposure of microbial metabolites or co-culturing living microorganisms in an enclosed lumen of organoids under static conditions favors the rapid overgrowth of bacteria, eventually resulting in the rupture and cell death of the organoids (Dutta & Clevers, 2017; J. Kim et al., 2020). The microfluidic perfusion of gut-on-chip offers the ability of the long-term exposure of the tissue to microbiota-associated metabolites under well-defined conditions. Perfusion media can be supplemented with defined single molecules of microbial metabolites up to filtrates of stool samples, single strain microorganisms, or defined microbial communities. To better recapitulate the *in vivo* situation, gut-on-chip platforms could be leveraged to mimic the conditions of different sides of the gut by use of adult stem cells derived from specific sections (i.e., intestine, colon, etc.) to generate side-specific tissue (Table 2). Colonization with oxygen-scavenging bacteria in combination with the continuous sensing and regulation of oxygen levels would allow the maintenance of physiological-relevant oxygenation levels and a co-culture with defined anaerobic and facultative-anaerobic bacteria under homeostatic conditions.

Table 2. Key references of selected gut-on-chip systems with important characteristics to recreate the intestinal environment

Reference	Model characteristics
Naumovska et al., 2020	plate-based pumpless monolayer cell culture system for hiPSC-derived epithelial cells
Wang et al., 2017; Wang, Kim, Gunasekara, et al., 2018	recapitulation of the crypt region and the stem cell niche of the intestine and colon within a hydrogel matrix
Kasendra et al., 2018; Kasendra et al., 2020; Sontheimer-Phelps et al., 2020	cultivation of organoid-derived polarized epithelial cells of the small intestine, duodenum, and colon-on-chip
Nikolaev et al., 2020	Hydrogel-confined crypt structures enable prolonged lifespan and improved cellular diversity of murine ISC-derived organoids
Workman et al., 2018	hiPSC-derived human intestinal epithelial cells form 3D crypt- and villus-like structures with self-patterned Paneth cells, goblet cells, enterocytes, enteroendocrine cells, transit amplifying cells, and Lgr5+ cells
Maurer et al., 2019	recapitulation of bacteria-fungal interaction in immunocompetent intestinal model
Shah et al., 2016	homeostatic colonization of epithelial cell layer with anaerobic

	bacteria under hypoxic conditions
Shin, Wu, et al., 2019	establishment of a hypoxic interface at an epithelial cell layer for co-culture with anaerobic bacteria
Jalili-Firoozinezhad et al., 2019	tunable oxygen gradients allow culture of obligate anaerobic bacteria and bacterial communities
Zhang et al., 2021	oxygen gradient allows stable co-culture of <i>Faecalibacterium prausnitzii</i> and identification of short chain fatty acid-related anti-inflammatory effects

Modeling human intestinal disease on-chip

Inflammatory diseases

Gut-on-chip models are offering a new avenue through which to study the pathophysiological mechanisms of gastrointestinal diseases. For example, patients suffering from Celiac disease (CeD), an autoimmune disease that affects about 1% of the human population, develop a strong inflammatory response causing serious damage to the small intestine upon the uptake of a gluten-containing diet (Caio et al., 2019). Still, the mechanistic details of the disease await further investigation before effective drugs can be developed. Moerkens et al. recently proposed an immunocompetent organ-on-chip model using both hiPSC and primary epithelial cells to better mimic the genetic background and environmental factors. Using this system, it is now possible to determine the disease onset in CeD patients (Moerkens, Mooiweer, Withoff, & Wijmenga, 2019). Similarly, gut-on-chip models have been used to study the disease mechanism of inflammatory bowel disease (IBD), which includes Crohn's disease (CD) and ulcerative colitis (UC), characterized by chronically relapsing intestinal inflammation and representing a worldwide healthcare problem with continually increasing incidence (Xavier & Podolsky, 2007). A 3D gut model based on primary patient-derived colonic epithelial cells forming a mucus layer was used to recapitulate prostaglandin E2-dependent mucus volume expansion via the activation of the Na-K-Cl cotransporter 1 ion channel (Sontheimer-Phelps et al., 2020). In a similar study, the pathophysiology of DSS-induced inflammation was also recapitulated in the presence of *E. coli* (Shin & Kim, 2018). DSS cessation quickly induced the recovery of barrier integrity, villus formation, and mucus production, indicating that this model might also be suitable to study processes of mucosal regeneration in IBD. The effects of microbial colonization were also investigated with a mixture of commensal *Streptococcus thermophilus*, Bifidobacteria, and Lactobacilli (VSL#3) as well as pathogenic enteroinvasive *E. coli* (EIEC). In this study, the authors were able to replicate inflammation-associated tissue damage and the invasion of EIEC. Furthermore,

they proved the growth-limiting effect of VSL#3 on EIEC colonization *in vitro* (H. J. Kim, Li, Collins, & Ingber, 2016). Recently, our group has been able to further replicate the protective characteristics of *Lactobacillus rhamnosus* against the tissue infiltration of the opportunistic yeast *Candida albicans* in an immunocompetent gut-on-chip model. In addition to peripheral immune cells, this model also contained functional mucosal macrophages and dendritic cells integrated into the epithelial tissue layer (Maurer et al., 2019). In contrast to conventional organoid cultures, chip-based ISC cultures enable long-term studies and perfusion with peripheral immune cells with superior accessibility and the continuous monitoring of key environmental factors to maintain and manipulate disease-related growth conditions. In combination with a homeostatic bacterial co-culture, these systems might serve in future studies as patient-specific tools to develop new therapeutics or probiotics that modulate the mucus barrier.

Infectious diseases

Gut-on-chip has further been used to study infectious diseases, including *Clostridioides difficile* infection (CDI), Enterohemorrhagic Escherichia coli (EHEC), and viral infections. Infection with *Clostridioides difficile* (formerly *Clostridium difficile*) is the most common cause of hospital-acquired gastrointestinal infections (Abbas & Zackular, 2020; Czepiel et al., 2019). While several animal models have been used to study CDI *in vivo*, the manipulation of infection processes and the direct assessment of host-bacterial interaction is challenging (X. Chen et al., 2008; Darkoh, Odo, & DuPont, 2016; Kuehne et al., 2010). Advanced gut-on-chip models have been described to investigate CDI. This model allows for the co-culture of primary epithelial and endothelial cells, with the addition of a complex microbiome under anaerobic culture conditions for several days (Jalili-Firoozinezhad et al., 2019). This is a promising translational tool to study the CDI infection process and associated damage in humans. As with CDI, infections with EHEC are frequent, with up 100,000 cases per year in the U.S. and is associated with several symptoms such as severe bloody diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS). Interestingly, the microbiota seem to be a critical determinant of susceptibility to EHEC infection. Studies in mice revealed that not only is a 100,000-fold higher dose of the bacterium required (10^7 microbes) but that the whole murine microbiome also needs to be depleted to induce symptoms. This pathology is quite different in humans, where as little as 10^2 microbes are sufficient to cause severe symptoms (Ikeda, Ito, & Honda, 2004; Keepers, Psotka, Gross, & Obrig, 2006; Mohawk & O'Brien, 2011). Tovaglieri et al. were able to recapitulate this phenomenon *in vitro* in a gut-on-chip model by co-culturing EHEC in the context of a murine or a human microbiome. The species-specific tissue damage induced by the human microbiome could

be verified. This model was also used to identify several metabolites related to or even causative of the high susceptibility of humans to EHEC (Tovaglieri et al., 2019). Finally, human enteroviruses are responsible for an estimated 10–15 million infections and at least 30,000–50,000 hospitalizations per year (Villeneuve et al., 2017). Viruses can replicate in the epithelial cells of the gastrointestinal tract and subsequently disseminate to secondary sites of infection, such as the respiratory tract (Racaniello, 2006). So far, no approved drugs for the treatment of enterovirus infections in humans besides polio exist (Racaniello, 2006). Villeneuve et al. used coxsackievirus B1 (CVB1) as a prototype enterovirus strain and established a human infection model based on a human gut-on-a-chip microfluidic device to study replication and infectious virus production *in vitro* (Villeneuve et al., 2017). With this model, they were able to follow the infection of epithelial cells, the release of inflammatory cytokines and the secretion of infectious virions. The model also permitted to reproduce the different susceptibility of epithelial and endothelial cell layers for viral infection by using separate microchannels, thus independently perfusing the vascular and epithelial sides of the polarized gut tissue. Gut-on-chip models have further just recently been used to study infection with human parasites. "Mini-intestines" fully accessible from the luminal side enabled colonization with *Cryptosporidium parvum*, an obligate parasite causing life-threatening diarrhea in immunocompromised adult hosts and in infants. The tissue model supported the full life cycle of the parasite, thereby allowing for the first time long-term studies in a primary cell-derived *in vitro* culture system (Nikolaev et al., 2020).

With the emergence of the SARS-CoV2 virus and the COVID-19 pandemic, the need for sophisticated models to study viral infection in human tissue has become even more urgent. Human small intestinal organoids have proven to be valuable tissue models to study virus docking to ACE-2 and its replication within enterocytes. Combining ISC-derived organoids with the advantages of organ-on-chip would offer additional options to manipulate the viral infection route and provide different parameters to faithfully recreate the intestinal microenvironment, prolong the culture of organoids with the improvement of cellular differentiation, and offer a variety of additional monitoring and readout techniques available for gut-on-chip systems (Lamers et al., 2020).

Colorectal cancer

The effects of SCFA released by probiotic *Lactobacillus rhamnosus* GG (LGG) bacteria on the growth of colorectal cancer cells was recently investigated in a version of the HuMiX model, a modular, microfluidics-based gut-on-chip model, which allows the co-culture of human and microbial cells and facilitates anaerobic culture conditions (Greenhalgh et al.,

2019). Symbiotic treatment with a high-fiber diet was simulated in this model, and the generation of organics and SCFAs was assessed. Strikingly, it was demonstrated that SCFAs and lactate production were altered by a simulated high-fiber diet compared to a reference diet medium containing only simple sugars. The simulated high-fiber diet caused an increased expression of oncogenes and proinflammatory signaling in the absence of LGG bacteria, whereas in the presence of LGG, both gene clusters were found to be significantly downregulated and associated with a reduced cell proliferation rate of primary colon rectal cancer cells. This study illustrates the capability of gut-on-chip systems to precisely dissect the individual aspects of the microbiota-host crosstalk at the metabolic level. Similar studies will hopefully further expand our insight into this complex relationship by identifying individual metabolic targets with potential as tailored pre- and probiotic-based therapeutic options. Uncovering individual beneficial metabolic profiles such as by the application of hiPSC-based organ models and individualized microbiota surrogates in personalized *in vitro* models would represent a major step forward in the treatment of IBD and IBD sequelae such as colorectal cancer.

Conclusions and outlook

The recent advances in the development of organ-on-chip technologies show not only the current advantages of these models but also highlight future possibilities in combination with stem-cell engineering and microfluidics. Such models have already proven to produce data similar to *in vivo* contexts in studies ranging from infectious disease modeling to drug metabolism. The benefits and wide range of applications of the gut-on-chip models can be attributed to their versatility and their capability to increment parameters of various types, from multiple cell types to medium gradients, flow mechanics, or global topography. Despite these numerous advances in the complexification of microfluidics systems, many requirements still need to be met in order to obtain models with an *in vitro* microenvironment that closely mimics its *in vivo* counterpart.

Gut-on-chip technology offers unique options by providing the technical basis for a reliable and guided differentiation of stem cells to human intestinal tissue offering combining the strengths of self-patterned stem cell-based organoids with the ability of precisely regulated biochemical and biophysical cues in a spatiotemporal manner. The manipulation of the microenvironment by tissue engineering and the guided growth of stem cells derived from patients will create a new angle for the dissection of host-microbiota interaction with scalable levels of complexity for the host and the microbiota. The ability to monitor and perturb the milieu in real time will help in understanding the dynamics and effects of changes in the microenvironment through the manipulation of isolated biochemical and biophysical cues.

Further, these systems will help in identifying the molecular and cellular targets of disease-related mechanisms for individual patients that cannot be resolved in similar detail in animal models. In the case of an organ-on-chip modeling of the gastro-intestinal tract, a more “complete” model would need to be composed of adjacent channels with human microvascular endothelium, tissue-resident immune cells, commensal or pathogenic bacteria, and the application of cyclic mechanical forces that mimic peristalsis-like deformations. The additional presence of muscle or nervous system cells could also contribute to creating a faithful model. Future efforts need to be undertaken in the development of more complex models that could be used for modeling human diseases, for drug development, and for further use in personalized medicine.

Clearly, organ-on-chip and in particular complex gut-on-chip models have several limitations. In contrast to conventional organoid cultures, their usability in high-throughput testing is limited. Further, ISC-based gut-on-chip as well as ISC-derived organoids represent reductionistic models, which do not reflect the full physiology offered by animal models. However, we are convinced that the combination of stem cell-based organoid culture and organ-on-chip will help pave the way to significantly expand our knowledge in the development and maintenance of the stem cell niche and its role in the onset of related diseases. It has already been demonstrated that this approach allows an increased organoid lifespan with higher cellular diversity and at the same time offers a multitude of options by which to manipulate and monitor essential biochemical and biophysical cues in tissue development and its interaction with the microbiota. Several challenges such as the uniformization of the systems and its standardization as “fit for purpose” and “ready to use” need to be overcome to allow researchers and non-organ-on-chip specialists easy access to this technology to allow its routine use in biomedical research.

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