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Modeling infectious diseases and host-microbe interactions in gastrointestinal organoids

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Abstract

Advances in stem cell research have allowed the development of 3-dimensional (3D) primary cell cultures termed organoid cultures, as they closely mimic the *in vivo* organization of different cell lineages. Bridging the gap between 2-dimensional (2D) monotypic cancer cell lines and whole organisms, organoids are now widely applied to model development and disease. Organoids hold immense promise for addressing novel questions in host-microbe interactions, infectious diseases and the resulting inflammatory conditions. Researchers have started to use organoids for modeling infection with pathogens, such as *Helicobacter pylori* or *Salmonella enteritica*, gut-microbiota interactions and inflammatory bowel disease. Future studies will broaden the spectrum of microbes used and continue to establish organoids as a standard model for human host-microbial interactions. Moreover, they will increasingly exploit the unique advantages of organoids, for example to address patient-specific responses to microbes.

Keywords

Organoid culture, Inflammatory bowel disease, Microbiota, Salmonella, Helicobacter, Rotavirus, Norovirus, Gastrointestinal disease.

Introduction

The human gastrointestinal tract is the prime interface for interactions with microorganisms. The healthy gastrointestinal tract is a finely tuned ecosystem of trillions of organisms, predominantly bacteria but also including bacteriophages, viruses and eukaryotes, collectively termed the microbiota. Food intake constantly introduces new microorganisms that can threaten this equilibrium. Perturbations in host-microbe interactions can lead to severe pathologies, such as inflammatory bowel disease (IBD), gastric ulcers and gastric cancer (reviewed in Abraham and Medzhitov, 2011; Suerbaum and Michetti, 2002), and therefore homeostatic mechanisms aim to minimize invasions of pathogens whilst maintaining beneficial interactions with the microbiota. These pathologies affect large numbers of people, with globally more than 700 000 deaths per year caused by gastric cancer alone, rendering the study of these diseases of prime importance (Ferlay et al., 2015). Recent advances in stem cell research have allowed the development of new model systems, termed "organoids" for their striking resemblance to organs. Organoids can be grown which represent many different organs, including the brain, liver, pancreas, lung, esophagus, stomach, small intestine and colon (reviewed in Clevers, 2016). Recent reviews have highlighted the general features of organoids (Clevers, 2016; Fatehullah et al., 2016; Huch and Koo, 2015; Lancaster and Knoblich, 2014) and also focused specifically on gastrointestinal stem cells and organoids (Dedhia et al., 2016; Werner et al., 2016). In this review, gastrointestinal organoids will be discussed with respect to their use in studying infectious diseases, host-microbe interactions and inflammation.

Gastrointestinal organoids and their advantages for studying host-microbe interactions

Organoids can be derived from two sources: (i) pluripotent stem cells (PSCs) or (ii) adult stem cells (ASCs) (Figure 1). PSCs can be obtained from embryos (embryonic stem cells, ESCs) or by reprogramming somatic cells (induced pluripotent stem cells, iPSCs). ASCs

reside in self-renewing tissues, such as the gastrointestinal tract, and either constantly renew the epithelium or can be activated to repair tissue upon damage. Both PSCs and ASCs have tremendous regenerative capacity, and in the past decade researchers have utilized increased understanding of the niche factors that favor stem cell maintenance to grow both types *in vitro* into organoids (reviewed in Clevers, 2016; Lancaster and Knoblich, 2014). In 2009, Sato and colleagues isolated intestinal crypts and the stem cells residing therein, placed them in a mouse sarcoma-derived extracellular matrix and supplemented the culture with three factors: the Wnt agonist R-spondin (RSPO), epidermal growth factor (EGF) and noggin (NOG), an inhibitor of bone morphogenetic protein (BMP). Under these conditions, intestinal stem cells proliferate and initially form small cysts which grow further into budding structures that can be expanded apparently without limits by splitting and re-seeding (Figure 1). The cultures contain highly polarized, differentiated cells of the intestinal lineages and exhibit an astonishing capacity to self-organize into domains that harbor the different cell types of both the crypt and villus domains (Sato et al., 2009). Mouse gastric organoids similarly reproduce the gastric epithelium *in vitro* (Barker et al., 2010). Following this, gastrointestinal organoids derived from human ASCs or PSCs were described soon after (Sato et al., 2011; McCracken et al., 2011; McCracken et al., 2014; Bartfeld et al., 2015), so opening new avenues for infection research.

Classically, infection research uses 2D cultures of cancer cell lines or animal models (Figure 2), although both of these systems have their drawbacks. Standard transformed cancer cell lines often fail to recapitulate even the very basic features of their *in vivo* counterparts: many cannot polarize or produce tissue-specific factors such as mucus. In addition, the cellular responses of cancer cell lines vary depending on whether they were grown in 2D or in 3D (Tung et al., 2011), and so results obtained in 2D monocultures may not necessarily reflect a 3D host response. Animal models may not always faithfully mimic human disease due to inter-species variations. Primary cell cultures and tissue explants are valuable additions to the modeling spectrum (Coron et al., 2009; Richter-Dahlfors et al., 1998), however their practicality is limited by the short-term nature of the culture and dependence on access to primary material. Organoids, on the other hand, contain highly polarized cells that differentiate into the cell lineages of the tissue of origin. For example, intestinal organoids contain fully mature goblet cells, enterocytes, Paneth cells and enteroendocrine cells (Sato et al., 2009) (Figure 3). Further, organoids can produce specific cell types that could not previously be generated or maintained in cell culture. Examples of these are gastrointestinal stem cells (Sato et al., 2009), intestinal M-cells (de Lau et al., 2012), Paneth cells (Sato et al., 2009), or gastric parietal cells (Noguchi et al., 2015; Schumacher et al., 2015a). Also, organoids can be directed to produce specific cell types; for example, gastric organoids can be grown so as to contain only cells of the gastric pit lineage or the gastric gland lineage (Bartfeld et al., 2015) (Figure 3). These features enable the study of interactions with specific target cells, for example the entry of *Salmonella* into M-cells, identification of specific target cells for enteric viruses, or stimulation of Paneth cell degranulation by infection.

There are several questions, which have not been possible to address using only 2D monocultures or animal models. For example, microorganisms that are restricted to humans could not be grown in pure cultures, human cell lines or animal models, were largely inaccessible for research. Although their presence could be detected by culture-independent techniques, such as DNA sequencing, experimental studies have not been possible. This still applies to a large part of the human microbiota (including bacteria, viruses, and eukaryotes) although many previously "unculturable" bacteria can now be

cultured (Browne et al., 2016). One example is norovirus, a common cause of diarrhea. A mouse model for norovirus exists, but due to species-specificity, human norovirus does not infect mice. Human norovirus was completely inaccessible to *in vitro* research, until the recent establishment of a B-cell model, which has enabled the virus to be cultured (Jones et al., 2014). However, *in vivo*, the virus infects the gastrointestinal epithelial cells, but attempts to infect epithelial cells in standard epithelial cell line culture have failed. Another example is rotavirus, which can be generally cultured, but isolation from primary samples is particularly difficult. These highly specialized microorganisms may need certain factors that are only present in differentiated human cells. Both viruses, norovirus and rotavirus have now been cultured in either 3D organoids or 2D cultures derived from organoids (see below), providing proof for the concept that the differentiated primary human cells present in organoids can provide essential factors for the infection and expansion of microorganisms.

One particularly clinically relevant application of organoids is the study of pathologies with high inter-individual variations, for example due to genetic risk factors, age, gender, etc. For example, *H. pylori* colonizes about 50% of all humans worldwide but only a fraction of the population develops gastric ulcers or gastric cancer (reviewed in Suerbaum and Michetti, 2002). Genome wide association studies (GWAS) have identified risk factors such as cytokines and pattern recognition receptors (reviewed in Wroblewski et al., 2010), but the mechanism underlying susceptibility is still unclear. Uniquely, organoids can be grown from seemingly every patient, which has allowed the generation of "living biobanks" amenable to phenotypic or drug screens (Fujii et al., 2016; van de Wetering et al., 2015; VanDussen et al., 2015). Such biobanks will enable researchers to identify and better understand risk factors in infection-associated pathologies such as gastric cancer and IBD, eventually paving the way for personalized treatments.

Gastric organoids and *Helicobacter pylori*

While the acidic environment of the stomach quickly kills most bacteria*, H. pylori* has evolved to colonize the gastric mucosa. Initial infection induces a rapid inflammatory response manifested as acute gastritis (Marshall et al., 1985). Over time, colonization can have two outcomes. In humans that do not develop chronic gastritis, *H. pylori* may have beneficial effects, as its presence is inversely correlated with reflux disease and the resulting metaplastic sequelae (reviewed in Plottel and Blaser, 2011). However, patients that develop chronic gastritis following the initial infection are at greater risk of developing gastric ulcers and gastric cancer (reviewed in Suerbaum and Michetti, 2002). While *H. pylori* can be cultured on bacterial plates and in liquid culture, proliferation ceases once the bacteria are added to standard eukaryotic cell cultures and the bacteria round up and die (Kusters et al., 1997; Segal et al., 1996). Further, cancer cell lines most probably represent a late stage of the disease process, which may be the stage of least relevance when considering the effect of *H. pylori* on carcinogenesis. Animal models are very useful, but they also have limitations (Solnick et al., 2016). As an alternative model lying between 2D cell lines and animals, several studies have now established *H. pylori* infection in gastric organoids.

To enable interaction with the apical side of the cell, which is also the natural site of infection, *H. pylori* are microinjected into the lumen of the organoids (Bartfeld et al., 2015; McCracken et al., 2014). Injected bacteria can adhere to the epithelium (Bartfeld et al., 2015) and target apical cell-cell junctions (Huang et al., 2015; Wroblewski et al., 2010). The bacteria are viable, as shown by re-culturing of *H. pylori* from organoids (Bartfeld et al., 2015; Bertaux-Skeirik et al., 2015) and even expand inside the organoids, suggesting that organoids may provide essential niche factors (Bertaux-Skeirik et al.,

2015). Epithelial cells in organoids secrete urea, which induces a chemotactic response in the bacteria and may contribute to the establishment of a replicative niche in the organoids (Huang et al., 2015). Attached bacteria can utilize their type four secretion system (T4SS) to inject at least one virulence factor, cytotoxicity associated gene A (CagA), into the host cell. In cancer cell line models, CagA binds to the host c-MET receptor and promotes a motogenic response (Churin et al., 2003). In gastric organoids, CagA also binds to c-Met and likely as a response, host cell proliferation is induced in a CagA-dependent manner (Bertaux-Skeirik et al., 2015; McCracken et al., 2014). Together, these studies establish organoids as a model for *H. pylori* infection by demonstrating that organoids reproduce important hallmarks of the infection.

Two studies have further used the unique features of organoids to study host-pathogen interactions. In one study, organoids have been differentiated into specific lineages of the stomach (Bartfeld et al., 2015). The gastric epithelium has a repetitive architecture with regular invaginations called the gastric glands. Similar to intestinal crypts, gastric glands harbor the stem cells. The region where a gland opens into the lumen of the stomach is called the gastric pit (Figure 3). When human ASC-derived organoids are differentiated so as to contain either cell lineages of the pit (MUC5AC-positive pit cells) or of the gland (Muc6-positive neck cells and chief cells) alone, cells of the gland region mount a stronger inflammatory response to *H. pylori* infection, measured as induction of cytokine mRNA (Bartfeld et al., 2015). This suggests a region-specific role of host cell factors involved in innate immune recognition of the bacteria, and further that these factors may play a role in protection of the stem cell-containing gland region.

The second study addressed the influence of *H. pylori* infection on parietal cells (Schumacher et al., 2015b). Parietal cells in the stomach produce acid and are the main source of sonic hedgehog (SHH), which in turn induces BMP production in the mesenchyme, so shaping the gland to form the stem cell niche. In humans, loss of parietal cells is a very early step in the process of gastric carcinogenic. Parietal cells have been refractory to previous culture attempts, but organoid cultures derived from mESCs (Noguchi et al., 2015), as well as short-term cultures of murine and human ASCs, harbor parietal cells (Schumacher et al., 2015a). *Shh* is induced in wild-type murine ASC-derived organoids infected with *H. pylori*, whilst organoids derived from mice with a parietal cell-specific deletion of *Shh* were unable to upregulate *Shh*, indicating that *H. pylori* infection induces *Shh* transcription specifically in parietal cells. This response was diminished by addition of nuclear factor kappa B (NF-κB) inhibitor VI, a form of resveratrol (Schumacher et al., 2015b). These results highlight the potential of organoids for infection research in the future.

Intestinal organoids, IBD, inflammation and microbiota

IBD is a chronic inflammatory disease of the gut affecting about 1.4 million patients in the United States alone. The two major forms of IBD are ulcerative colitis, which is restricted to the colon, and Crohn's disease, which can affect several sites of the body. While the cause of the disease is not fully understood, genetic evidence points to the importance of the microbiota and the host's inflammatory response during pathogenesis (reviewed in Abraham and Cho, 2009; Abraham and Medzhitov, 2011; Xavier and Podolsky, 2007). In the gastrointestinal tract, the microbiota constantly interact with the epithelium, not only by direct contact but also *via* metabolites or other secreted factors. For example, metabolites such as the short chain fatty acid butyrate are produced by the microbiota and taken up by the host. In addition, the microbiota shed molecules that are unique to each kingdom (bacteria, fungi or viruses) and can be recognized as foreign by the host. These factors are called pathogen or microbe associated molecular patterns (PAMP/MAMP) and are recognized by host pattern recognition receptors (PRR). GWAS

reports have associated IBD with genes that shape the inflammatory response to the microbiota, such as nucleotide oligomerization domain 2 (*NOD2*), autophagy genes and inflammatory regulators, such as the IL23 receptor (*IL23R*). Although many reports point to a role of the intestinal epithelium in IBD, current evidence is primarily derived from animal studies and observational studies. Organoids have great potential to be an ideal experimental model as they are composed of patient-derived, primary human epithelial cells, and are also accessible to experimental approaches. Specifically, organoids may provide a system that is able to address if an intrinsic defect in the intestinal epithelium contributes to disease pathogenesis. Recently, a biobank of organoids has been established from samples taken from throughout the gastrointestinal tract of IBD patients (VanDussen et al., 2015). Organoids from IBD patients have been sequenced and the results have confirmed known risk factors (Mokry et al., 2014). As well as overall changes in the microbiota, several specific pathogens (such as *Clostridium difficile,* see below) have been associated with the disease, although a causal relationship has not yet been confirmed (reviewed in Abraham and Cho, 2009).

Pattern recognition

Pattern recognition is a lynchpin of interaction with the microbiota, because usually the activation of PRRs induces the central pathway of the innate immune response, activation of NF-κB, and activation of NF-κB in turn leads to transcription of inflammatory cytokines such as tumor necrosis factor alpha (TNFα), IL1β or IL8. RNA analysis of laser captured cells and subsequent immunodetection showed that PRR expression is highly restricted *in vivo*, with the deregulation of PRRs being associated with IBD (reviewed in Abreu, 2010). However, until recently, functional studies could only be carried out using transformed monotypic (cancer) cell lines or short-term cultures of isolated epithelial cells, and there was debate surrounding the interpretation of these results, given that the models used may not have fully represented the *in vivo* situation (Abreu, 2010). Recently, several studies have used organoids to investigate the presence of PRRs, their function and the effect of their stimulation. Farin and colleagues showed that murine intestinal organoids express several PRRs (toll like receptors [*Tlr*] *2,3,7,8,9, Md-2* and *Cd14*). Surprisingly, the classical PAMP stimuli for these PRRs did not induce NF-κB signaling as measured by NF-κB target gene activation (Farin et al., 2014). Notably however, the same PAMPs induced NF-κB target gene transcription in two cancer cell lines, highlighting the difference between cell lines and organoids (Farin et al., 2014). Another group confirmed the lack of response to TLR4 and TLR2 stimulation, but reported upregulation of proinflammatory cytokines in response to TLR3 stimulation (Davies et al., 2015). Although TLRs have been reported to be expressed on Paneth cells (reviewed in Abreu, 2010), PAMPs did not induce the degranulation of Paneth cells in organoids, independent of whether the PAMPs were added to the basolateral or apical side (Farin et al., 2014). This indicates that although PRRs are expressed in intestinal organoids, classical PRR signaling is largely silenced, probably preventing stimulation by the microbiota. By contrast, Paneth cell degranulation can be stimulated by interferon gamma (IFNγ), a proinflammatory cytokine commonly secreted by immune cells (Farin et al., 2014), so placing a layer of immune cell control upstream of parietal cell-mediated defense.

If PRRs are expressed in the intestinal epithelium but do not induce signaling leading to the transcription of proinflammatory cytokines or to Paneth cell degranulation, what could be the other effects of stimulation? Previous studies in mice have elegantly linked PRR signaling to epithelial homeostasis (Nenci et al., 2007; Rakoff-Nahoum et al., 2004). Organoid studies could now show that PAMPs have a direct effect on epithelial

regeneration. Nigro and colleagues isolated intestinal crypts and added PAMPs to the culture when seeding them into organoid growth media. Two of the factors, peptidoglycan and muramyl-dipeptide (MDP), both components of bacterial cell walls which bind to NOD1 and NOD2 receptors, respectively, induced increased organoid outgrowth. Stem cells expressed NOD2, and MDP specifically stimulated stem cell survival. When the epithelium was damaged by doxorubicin in mice, MDP stimulation induced proliferation and this was abrogated in *Nod2*$^{-/-}$ knockout mice (Nigro et al., 2014). This links microbial factors directly to epithelial regeneration with NOD2, one of the genes associated with IBD, at the center of the interaction.

Other PRR stimulation, such as through TLR3, may have more subtle effects, as organoids become more cystic, have fewer budding structures and exhibit mildly decreased survival (Davies et al., 2015). The underlying mechanisms of PRR-induced survival or phenotypic changes remain to be elucidated.

Bacterial metabolites and hypoxia

Bacterial metabolites, for example short chain fatty acids such as butyrate, are taken up by the host cells and used as an energy source. A decrease in butyrate-producing bacteria has been associated with IBD (reviewed in Miquel et al., 2013). Culture supernatants of bacteria and isolated metabolites, especially butyrate, induce wide-ranging changes in gene expression in intestinal organoids (Lukovac et al., 2014). In a recent study, researchers analyzed the effect of 92 bacterial metabolites and PAMPs on colonic organoids. Of these, butyrate strongly inhibited the proliferation of mouse colon organoids, while PAMPs had little effect (Kaiko et al., 2016). Using the unique capability of ASC-derived organoids to differentiate, researchers grew organoids either in conditions that support stemness or in conditions which promote differentiation to colonic enterocytes. Butyrate selectively inhibited the growth of stem cells but not of enterocytes. The authors hypothesized that, in a healthy colon, the regular architecture of the tissue shields the stem cells from the microbial butyrate: enterocytes at the surface of the colon metabolize butyrate, thus creating a gradient with the lowest concentration of butyrate at the base of the colonic crypts, where the stem cells reside. Indeed, in situations where the colonic architecture was disrupted, butyrate impaired regeneration *in vivo* (Kaiko et al., 2016).

Enterocytes metabolize the butyrate from commensal bacteria to CO_2, leading to low oxygen levels of < 1% (hypoxia). Low oxygen levels stabilize the hypoxia inducible factors *HIF1α* and *HIF2α*, which in turn upregulate barrier functions of the intestinal epithelium (Kelly et al., 2015). Higher levels of HIFs are observed in IBD (reviewed in Eltzschig and Carmeliet, 2011). Normally, cell culture is carried out at normoxia (20% O_2). Transfer of human iPSC-derived intestinal organoids grown in normoxic conditions to hypoxic conditions for 24h induces TNFα transcription (Xue et al., 2013), similar to hypoxia induced by intestinal reperfusion *in vivo* (Chen et al., 2003). In organoids, this TNFα transcription was inhibited by the HIF2α inhibitor piplartine or acriflavine (Xue et al., 2013).

Anaerobic bacteria

In the healthy gut, a steep O_2 gradient from the anaerobic lumen to the hypoxic epithelium is established. Because organoids grown in 3D have a sealed lumen and a mucus layer, it may be possible that an oxygen gradient is established that may be closer to the *in vivo* situation than in 2D cell lines, and thus may more accurately model hypoxia and changes in oxygen levels. In addition, many members of the gastrointestinal microbiota are obligate anaerobic bacteria and their anaerobic lifestyle hinders *in vitro* experimentation with 2D cell lines grown in standard 20% O_2. Two anaerobic bacteria, *Bacteroides thetaiotaomicron* (*B. thetaiotaomicron*), a member of the healthy microbiota,

and the anaerobic pathogen *Clostridium difficile* (*C. difficile*), have each been injected into organoids (Engevik et al., 2013; Engevik et al., 2015; Leslie et al., 2015). While the *B. thetaiotaomicron* study did not address the question of bacterial survival, authors of the *C. difficile* study re-cultured bacteria from organoids after 12 hours of infection, indicating that anaerobes can survive for 12 hours in organoid culture (Leslie et al., 2015). This groundwork demonstrates that organoids can be used for studies requiring the culture of anaerobic bacteria. It will be important to more closely evaluate O_2 levels and viability of bacteria in the organoids, and so it is likely that future studies will explore the use of organoids for the study of the anaerobic microbiota of the gut.

Intestinal organoids and pathogens - bacteria, viruses and parasites

C. difficile is not a normal part of the human microbiota but their spores are ubiquitously present, for example in soil, and can be taken up with food. Certain conditions, such as an imbalance in the microbiota due to the use of antibiotics, can provide a potential niche for the outgrowth of *C. difficile*. Toxins produced by the bacterium then cause mild to severe gastroenteritis, but can also lead to severe complicated pseudomembranous colitis, characterized by inflammatory lesions and pseudomembranes in the colon (reviewed in Rupnik et al., 2009). During *C. difficile* infection of organoids, bacterial toxins could also be shown to inhibit barrier function, as assessed by the retention of FITC-dextran (Leslie et al., 2015), and diminish expression of Na+/H+ exchanger 3 (NHE3), which may influence gut microbiota composition (Engevik et al., 2013; Engevik et al., 2015).

Salmonella enteritica serovar Typhimurium (*S.* Typhimurium) causes self-limiting gastroenteritis in humans and systemic disease in susceptible mice. As a facultative intracellular bacterium, *Salmonella* uses virulence factors encoded in the *Salmonella* pathogenicity islands SPI-1 and SPI-2 to invade and replicate inside host cells, including macrophages and epithelial cells (reviewed in Figueira and Holden, 2012). Injection of mouse ASC-derived intestinal organoids, as well as human iPSC-derived intestinal organoids, with *S*. Typhimurium resulted in the upregulation of host cell genes, including typical proinflammatory cytokines such as IL-1β, TNF and IL-8 (Forbester et al., 2015; Zhang et al., 2014). Intracellular bacteria could be detected in iPSC-derived organoids, with cellular invasion dependent on the known bacterial invasion protein A (*invA*) (Forbester et al., 2015). Defensins secreted by host cells, such as macrophages or epithelial Paneth cells, have the potential to kill *Salmonella*, but the bacterial gene *phoP* confers partial resistance to defensins (Fields et al., 1989). Until recently, the interaction of *Salmonella* with Paneth cells could not be studied *in vitro* due to a lack of appropriate model systems. Mouse ASC-derived intestinal organoids have functional Paneth cells that can be stimulated to degranulate and secrete antimicrobials into the lumen of the organoids (Farin et al., 2014). Indeed, contrary to standard cell cultures, wild type organoids largely inhibit the growth of *S*. Typhimurium even after 20 hours of infection. However, if maturation of α-defensins is abrogated by ablation of matrix metalloproteinase 7 (*Mmp7*) in organoids generated from *Mmp7*$^{-/-}$ mice, *S.* Typhimurium can replicate. This is even more apparent in *PhoP* mutants (Wilson et al., 2015).

Gastrointestinal viruses cause a large part of the yearly estimated 1.7 billion cases of acute diarrhea (Finkbeiner et al., 2012). Next to norovirus, rotavirus, a double-stranded RNA virus of the family *Reoviridae,* is the most common cause of diarrhea in young children. This virus can be studied in the laboratory, as there are culture-adapted strains. However, to obtain human rotavirus from fecal samples, the virus must be adapted to cell culture by several passages in primary African green monkey kidney cells. The use of organoid cultures would be ethically preferable to primary monkey cells. Indeed, PSC-derived intestinal organoids support the replication of the virus, as

judged by PCR of viral RNA and immunofluorescence of viral proteins (Finkenbeiner et al. 2012). Future experiments are required to demonstrate if this also leads to the production of infectious virus particles, which would ultimately prove that organoids can maintain rotavirus obtained directly from patients. In contrast, a cell culture-adapted strain of rotavirus has been shown to efficiently replicate in organoids, specifically in ASC-derived human small intestinal organoids. The lab-strain enters and destroys cells, which is observed as a typical cytopathic effect. Infection also produces infectious progeny, judged by a standard infectivity assay on a cell line (Saxona et al. 2016). Animal rotavirus induced decreased cytopathicity, indicating that it exhibits species-specificity in organoids similar to that seen *in vivo* (Saxona et al. 2016). The group further used the unique possibility of organoids to differentiate into specific cell types and found that the percentage of infected cells rose from 12% to 37% upon differentiation. This highlights the advantages of organoids to provide cell types that are highly accessible for viral infections.

The use of gastrointestinal organoids for protozoan parasites is still in its infancy. However, similar to the systems used for viruses, organoids can be expected to provide optimal target cells and enable culture from a broader range of patients than currently possible (Klotz et al., 2012). For example, *Toxoplasma gondii* is an obligate intracellular parasite that causes toxoplasmosis, a symptomless or mild illness in healthy individuals, that can cause serious encephalitis in immuno-compromized patients or when transmitted to the fetus during pregnancy. ASC-derived human intestinal organoids have been infected with *T. gondii* as shown by immunofluorescence (Klotz et al., 2012), and thus may be a good model for this infection. Future studies will be required to establish and characterise organoids as new models for eukaryotic pathogens, including parasites and fungi.

Current limitations, complex tissue engineering or simplified 2D layers from organoids

Development of organoid cultures has provided researchers with new model systems that fill the gap between standard 2D culture and animal models (Figure 2). Although organoid technology is still evolving, there are some shortcomings that need to be addressed in the future. Currently, a variety of systems are available and each system uses different culture conditions, which may impact experimental outcomes. There are a lack of studies that analytically compare systems to help decide which model is best suited for a particular scientific question. Also, genome-wide data, more functional assays, and single cell analysis is needed to describe the features of cells in organoids in more depth, for example to better understand, how well a particular gut segment is represented, which exact cell types are present and how well they are differentiated. Furthermore, a more detailed description is required of how well organoid properties are retained over time in long-term culture. For bacterial infection studies, it would be particularly desirable to identify an alternative for the current extracellular matrix (Matrigel), as it contains gentamicin, which needs to be removed before infection studies.

Current organoids are a reduction model of organs, as organoids are comprised of only epithelium and mesenchyme, or can even be purely epithelial, and so they lack immune cells, neurons and vascularization. While this can certainly also be seen as an advantage by allowing the study of epithelial interactions without possible confounding effects from other cell types, it can also be seen as a limitation. Co-cultures and tissue engineering approaches will generate more complex stem cell-derived tissues which will allow the study of interactions with immune cells or other cell types (reviewed in Ramanan et al., 2014). For example, recently an exciting 3D model of human colon has

been published, repopulating decellularized colonic mucosa with modified organoid-derived epithelial cells, endothelial cells and myofibroblasts (Chen et al., 2016). This and other engineered tissues may pose more complex models for infection biology in the future.

On the other side of the spectrum, infection biologists may prefer to simplify the model and use organoids merely as a source of 2D culture material, rendering the addition of microorganisms to the medium on top of an epithelial cell layer more convenient than microinjection (Bartfeld and Clevers, 2015; Schlaermann et al., 2016; VanDussen et al., 2015). Recently, a breakthrough was achieved by using 2D cultures from ASC-derived intestinal organoids: after decades of failed attempts to culture human norovirus in epithelial cell lines, Ettayebi and colleagues demonstrated that the virus can infect organoid-derived enterocytes (Ettayebi et al., 2016). For this, ASC-derived organoids were cultured according to standard methods (Sato et al., 2011), then disrupted into single cells and seeded in 2D layers (VanDussen et al. 2015). Fecal samples collected from patients harboring norovirus were filtered and added to the cells for one hour. The virus was then removed and enterocyte differentiation was induced by adding the appropriate medium to the cells. Notably, using this and similar infection protocols, norovirus cannot infect standard cell lines. However, the virus was detected by immunofluorescence inside the organoid-derived enterocytes and production of new virus particles was detected by RT-PCR. Most importantly, the virus was also passaged on the enterocytes, demonstrating that the infection yields infectious progeny (Ettayebi et al., 2016). Interestingly, the infectivity is partly dependent on the presence of bile, a natural content of the intestinal lumen. Several strains, for example GII.3, will only infect cells in the presence of or after pre-treatment with bile. Indeed, only one particularly infectious strain which is responsible for major outbreaks worldwide, GII.4, infects enterocytes in the absence of bile, although even for this strain infectivity is enhanced by bile (Ettayebi et al., 2016). It is not presently clear which component of the bile aids the infection. Using organoids from a range of patients, the study further verified epidemiological data that the presence of a functional fucosyltransferase gene (*FUT2*) correlates with susceptibility to infection with norovirus GII.4 (Ettayebi et al., 2016). This study impressively demonstrates that organoids can provide host cells for microorganisms that were previously unamenable to culture. This new model for human norovirus will now enable research to identify the receptor for the virus, the active component of the bile and possible therapies to prevent or treat infection.

Several bacterial studies have also used 2D cultures for their simplified handling and increased accessibility for infection. *H. pylori* can translocate the bacterial CagA protein into 2D layers of human gastric cells derived from organoids (Schlaermann et al., 2016). If human intestinal crypts are seeded into 2D in conditions supporting M cell differentiation (de Lau et al., 2012), *S*. Typhimurium preferentially infects M cells (Rouch et al., 2016). Organoid cultures from different sites of the intestine, such as duodenum, jejunum and ileum, retain their region-specific markers in 3D (Middendorp et al., 2014). Enteropathogenic, enteroaggregative and enterohemorrhagic *Escherichia coli* (EPEC, EAggEC and EHEC) differentially adhere to 2D layers derived from ileal or rectal organoids, implying the presence of region-specific adherence factors. These results indicate the applicability of 2D layers for infectious studies. It is, however, unclear as to whether 2D cell lines have similar levels of differentiation and domain organization as 3D cultures. Also, in the protected lumen of a 3D structure, conditions may differ from those in 2D layers, for example in regard to O_2 levels or the accumulation of mucus or metabolites such as butyrate or urea. These are factors, which could potentially

influence the progression of infection, and thus 2D cultures may not provide the level of insight that could be obtained in 3D (Tung et al., 2011).

Concluding remarks and outlook

The use of organoids in infection biology is a new but rapidly expanding field. It can be expected that organoids will become a standard tool to verify and follow up on results obtained in other models. Future studies will broaden the spectrum of infectious agents investigated, be it commensals, pathogenic bacteria, viruses, parasites or fungi. Briefly looking beyond gastroenterology, fascinating brain organoids have been used to model Zika virus infection. The virus preferentially infected and replicated in neural progenitors, induced cell death and decreased proliferation, leading to reduced neuronal cell layers and thus phenocopying the microencephaly caused by the virus in human fetuses (Dang et al., 2016; Garcez et al., 2016; Tang et al., 2016). Other organoids such as lung (Dye et al., 2015; Huang et al., 2013), liver (Huch et al., 2015; Takebe et al., 2013), kidney (Takasato et al., 2015) and fallopian tube organoids (Kessler et al., 2015) may represent ideal models for infections of the respective tissues, such as respiratory virus, malaria parasite, biofilm-producing *E. coli* or *Chlamydia* infection, respectively.

Future studies will use the unique features of organoids, such as the presence of specific cell types, to better understand infectious and inflammatory diseases. With the creation of organoid biobanks, a remarkable resource is being generated that opens new avenues to study inter-individual factors influencing host-microbe interactions. In highly prevalent infections, such as *H. pylori*, it will be of prime importance to identify which patients are at risk of disease development. By combining the currently established infection models with the newly generated biobanks, researchers now have the means to address patient-specific responses to verify previously identified risk factors and analyze their mechanism. Combining the patient-specific models with the use of organoids for drug screening will enable researchers to move forward in the development of personalized medicine and find new therapies for infectious diseases, including infection-associated cancers.

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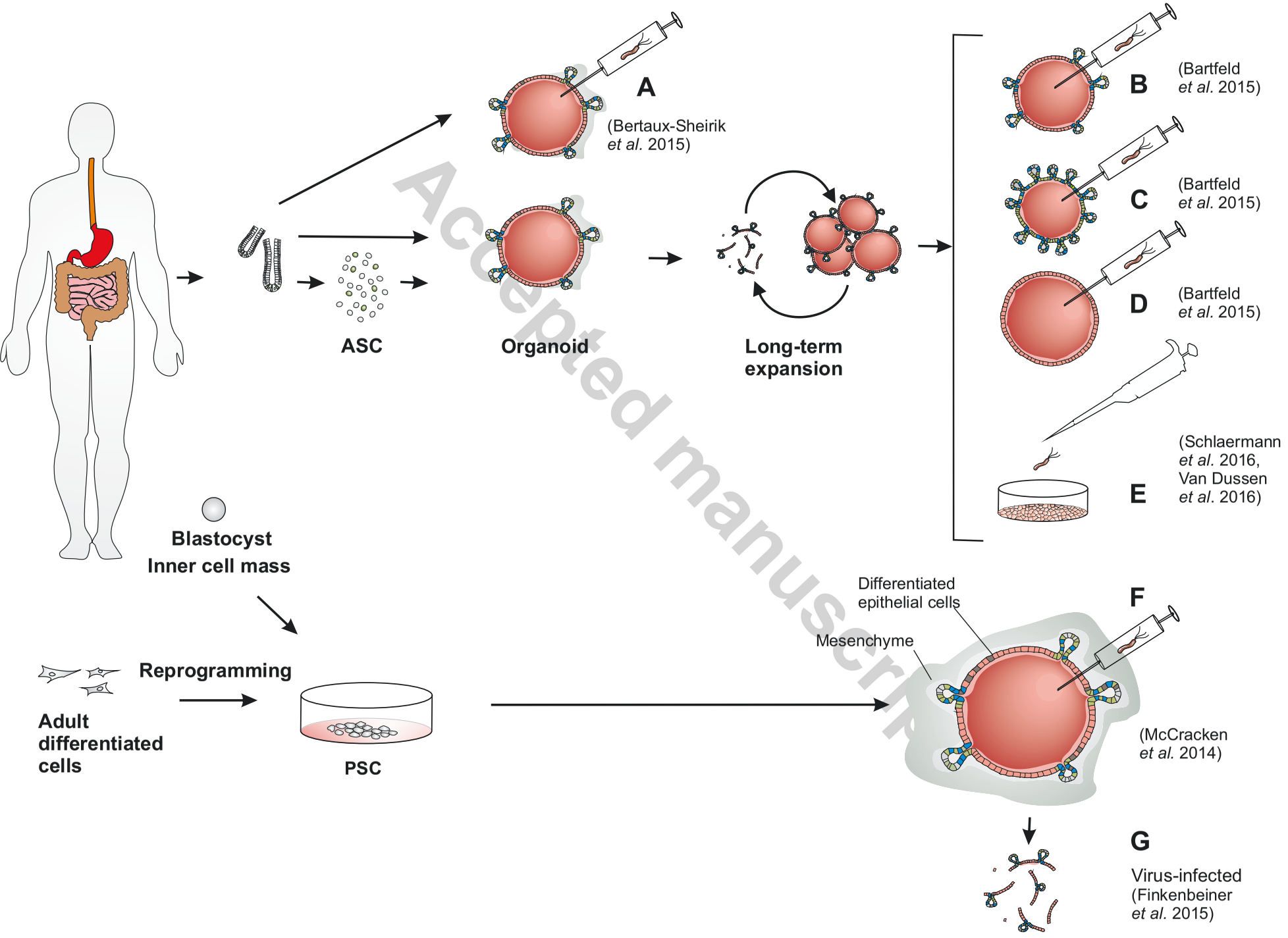
Figure 1: Gastrointestinal organoids can be grown from adult stem cells (ASCs) or pluripotent stem cells (PSCs). After initial seeding, some ASC-derived cultures contain a mesenchymal compartment (A). Long-term culture of ASC-derived, purely epithelial organoids provides infection researchers with unlimited access to primary cells (B-E). Long-term human cultures contain many different lineages of the original tissue (B) and can be directed into specific lineages (C/D) (Bartfeld et al., 2015; Sato et al., 2011). PSC-derived organoids contain a mesenchymal compartment and differentiated epithelial cells (F). Bacteria are commonly microinjected into organoids (A, B, C, D, F), but can also be added into the supernatant of organoid-derived 2D cultures (E). Viral infection has been established using mechanical disruption (G). Examples of studies using human organoids for infection are given in the figure; please see main text for details and examples for mouse organoids.

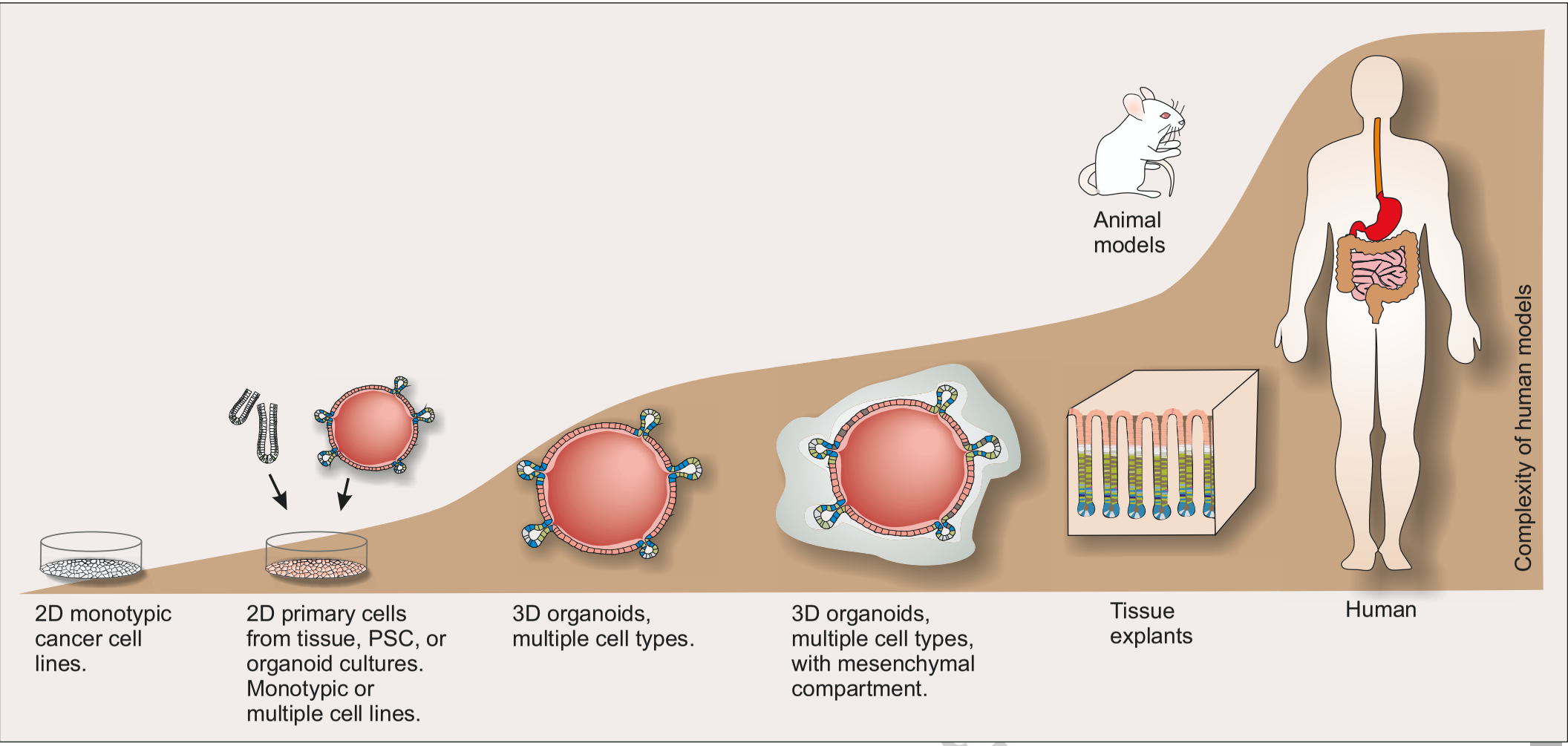
Figure 2: Mimicking the 3D organization and architecture of tissue, human organoids complement the model spectrum between 2D monotypic cancer cell lines, tissue explants and full organisms.

Figure 3: Organoids are primary, non-transformed, organized multicellular structures. They contain differentiated cells of the tissue they originate from. Cell differentiation can be directed to specific cell types, such as M-cells.

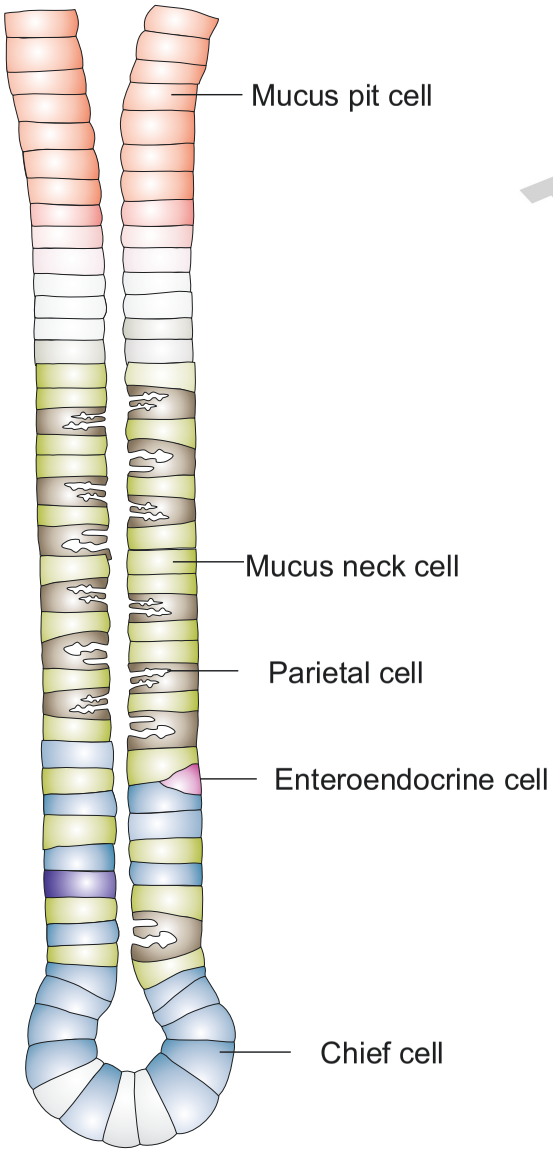
Highlights

- Organoids are now used widely to study infection biology, interaction with the microbiota and associated diseases such as IBD.
- Organoids contain differentiated host cells crucial for interaction with microorganisms.
- Organoids provide host cells for microorganisms which were previously difficult to culture.
- Simplified 2D layers and engineered tissues complete the spectrum of new model systems.

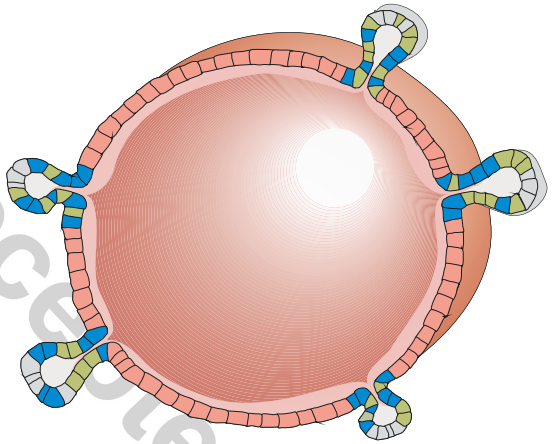



Animal models
Complexity of human models
2D monotypic cancer cell lines.
2D primary cells from tissue, PSC, or organoid cultures. Monotypic or multiple cell lines.
3D organoids, multiple cell types.
3D organoids, multiple cell types, with mesenchymal compartment.
Tissue explants
Human

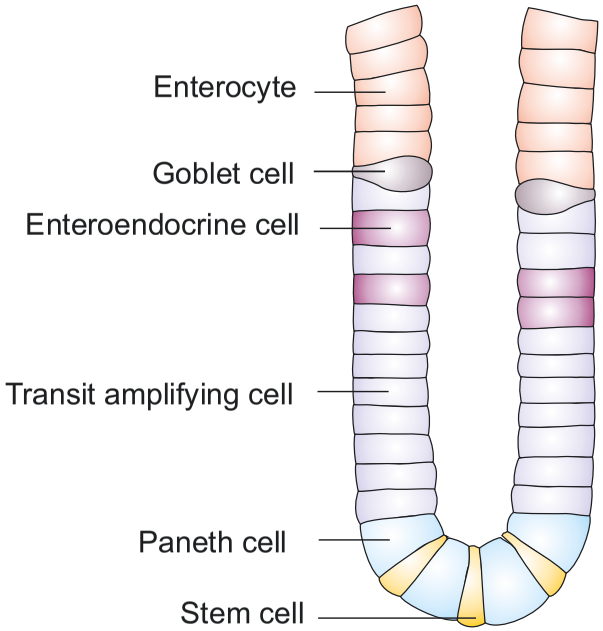
Gastric corpus unit



Organoid



Small intestinal crypt



Generation of indicated cell lines is described in:

Intestinal organoids
Sato *et al.* 2009
Sato *et al.* 2011
McCracken *et al.* 2011
De Lau *et al.* 2012

Stomach organoids
Barker *et al.* 2009
McCracken *et al.* 2014
Bartfeld *et al.* 2015
Schumacher *et al.* 2015a
Noguchi *et al.* 2015