Review

Focus: Induced Pluripotency & Cellular Reprogramming

Generating human intestinal tissues from pluripotent stem cells to study development and disease

Katie L Sinagoga¹ & James M Wells¹ 2 *

Abstract

As one of the largest and most functionally complex organs of the human body, the intestines are primarily responsible for the breakdown and uptake of macromolecules from the lumen and the subsequent excretion of waste from the body. However, the intestine is also an endocrine organ, regulating digestion, metabolism, and feeding behavior. Intricate neuronal, lymphatic, immune, and vascular systems are integrated into the intestine and are required for its digestive and endocrine functions. In addition, the gut houses an extensive population of microbes that play roles in digestion, global metabolism, barrier function, and host–parasite interactions. With such an extensive array of cell types working and performing in one essential organ, derivation of functional intestinal tissues from human pluripotent stem cells (PSCs) represents a significant challenge. Here we will discuss the intricate developmental processes and cell types that are required for assembly of this highly complex organ and how embryonic processes, particularly morphogenesis, have been harnessed to direct differentiation of PSCs into 3-dimensional human intestinal organoids (HIOs) in vitro. We will further describe current uses of HIOs in development and disease research and how additional tissue complexity might be engineered into HIOs for better functionality and disease modeling.

Keywords endoderm; intestinal development; organoids; stem cells; tissue engineering

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Introduction

The human intestine averages 8 m in length (Hounnou et al., 2002) with an epithelial surface area of 250 qm², roughly equivalent to the area of a tennis court. Epithelial surface area is amplified by the presence of villi, which are finger-like projections that protrude into the lumen of the intestine and microvilli, which are hair-like structures on the surface of absorptive epithelial cells (enterocytes). The epithelial morphogenesis that occurs in late embryonic and early postnatal stages also creates the crypts of Lieberkühn at the base of the villi. Crypts contain a specialized subset of intestinal cells including stem and progenitor cells that constantly renew the intestinal epithelium. In the mouse, the entire intestinal epithelium is turned over once every 5 days. There are two major cell types in the intestinal epithelium: the secretory cells and absorptive enterocytes. Comprising 90% of the epithelium, the enterocytes are responsible for the uptake and subsequent distribution of nutrients to the blood vasculature. The three secretory lineages are the hormone-secreting enteroendocrine cells (EECs), the mucus-producing goblet cells, and the Paneth cells in the crypt, which secrete antimicrobial peptides and help maintain the stem cell niche (Fig 3).

The intestine is comprised of two major segments, the small and large intestines. Proximally located, the small intestine is further subdivided into the duodenum, jejunum, and ileum, while the distal large intestine includes the cecum and colon. Morphologically, the proximal intestine can be distinguished by long, leaf-like villi, which become shorter and flatter in the colonic epithelium. These morphological changes reflect the unique functions that different segments of the intestine have in the breakdown of food and the uptake of nutrients. For example, the duodenum is responsible for acid neutralization and enzymatic breakdown of chyme from the antrum, while the jejunum is the main site of total nutrient absorption. More distal, the ileum is responsible for bile reabsorption and the colon is the main site of short-chain fatty acid (SCFA) production. These examples are only a few of the many roles each region plays in digestion and absorption of nutrients. For a more comprehensive look at the digestive physiology of the intestine, refer to Gastrointestinal Anatomy and Physiology: The Essentials (Reinus & Simon, 2014). The unique structure and function of each region of the intestine only highlights the complex underlying cellular differences. These include region-specific EEC subtypes as well as nutrient transporters and sub-epithelial populations that aid in the function of each region.

Infection, cancer, inflammation, intestinal motility disorders, and malabsorptive diarrhea are among the most prevalent human maladies. As a result, there have been many studies in model organisms aimed at understanding the development, homeostasis, regeneration,
and pathologies of the intestinal tract. However, until recently there were no in vitro systems available that adequately modeled the development and pathophysiology of the gut. In the past few years, several approaches have been developed to generate human intestinal tissue in vitro. One approach used intestinal biopsies from patients, from which are derived from primary cultures of intestinal epithelium (Sato et al., 2011), which has been extensively reviewed elsewhere (Sato & Clevers, 2013). This review focuses on another approach using directed differentiation of human pluripotent stem cells (PSCs) into three-dimensional, human intestinal organoids (HIOs) (Spence et al., 2011b). HIOs, generated either from embryonic stem cells or from induced pluripotent stem cell lines (iPSCs), are being used to study many aspects of human intestinal development, including cell–cell interactions, the stem cell niche, patterning of the intestine, the differentiation of multiple cell types from intestinal stem cells (ISCs), and intestinal diseases. Below we will outline our current knowledge of intestinal development, function, and disease and discuss how HIO-based models are currently being used to understand these processes in a human context.

Development of the intestines in vivo and in pluripotent stem cell cultures

Endoderm formation

The directed differentiation of PSCs into intestinal tissue in vitro was achieved by temporally manipulating the signaling pathways that are involved in normal intestinal development in vivo. This begins with formation of the endoderm germ layer during gastrulation (see reviews Zorn & Wells, 2007, 2009; Spence et al., 2011a). At the molecular level, endoderm formation is a highly conserved process in mouse, fish, frog, and humans, requiring the TGF-beta family member Nodal. Nodal signaling launches a cascade of gene expression at e13.5 (Gregorieff et al., 2004), a phenotype that is similar to deletion of Cdx2 in the mid-gestation embryo (Grainger et al., 2010). Deletion of Wnt5a caused shortening of both the small and large intestines as well as disruption of apical–basal polarity of the epithelium (Yamaguchi et al., 1999; Cervantes et al., 2009). BMP ligands that are expressed in the posterior mesoderm in a signaling loop with Sonic Hedgehog from the endoderm to regulate posterior Hox13 gene expression in the hindgut of chick embryos (Roberts et al., 1995). Lastly, FGF10 signaling, mediated through FGFR2IIb, is involved in the development of several regions of the intestine and embryos with defective FGF10 signaling have cecal defects and colonic atresia (Burns et al., 2004; Sala et al., 2006). It should be possible to manipulate these pathways to regulate regional identity of HIOs, although this has not yet been reported.

Intestinal morphogenesis in vivo and in HIO cultures: the importance of 3-dimensional structure for function

The initial transition from a two-dimensional sheet of DE to a three-dimensional tube occurs shortly after gastrulation. While BMP, Wnt ligands are expressed in the posterior of gastrula stage vertebrate embryos and the role of Wnt/β-catenin signaling at this stage of development is to promote a posterior fate, while inhibiting an anterior fate in the endoderm (McLln et al., 2007; Sherwood, 2009). Likewise, FGF and BMP ligands that are expressed in the posterior act on the endoderm to promote a posterior fate in chick and mouse embryos (Roberts et al., 1995; Wells & Melton, 2000; Tiso et al., 2002; Kumar et al., 2003; Dessimoz et al., 2006; Rankin et al., 2011). WNT and FGF act in part by regulating the transcription of the Caudal homeobox (Cdx) family of transcription factors that play an evolutionarily conserved role to specify embryonic posterior fate. The Cdx family of genes in vertebrates (Cdx1, Cdx2, Cdx4) work in tandem with FGF signaling to promote the expression of posterior Hox genes Hoxa7 and Hoxb9 (Pownall et al., 1998; Ho et al., 1999; Zorn et al., 1999; Ehrman & Yutzey, 2001; Bel-Vialar et al., 2002; Spence et al., 2011b; Chen et al., 2013). These data indicate that Wnt/β-catenin and FGF signaling regulate the posteriorization of endoderm through activation of the Cdx genes.

The pathways that govern posterior fate of endoderm in embryos can also direct human pluripotent stem cell-derived definitive endoderm into a posterior fate (McCracken et al., 2011; Spence et al., 2011b; Chen et al., 2013). In one example, the combined activation of WNT and FGF was sufficient to direct human definitive endoderm (DE) cultures to adopt a hindgut identity as evidenced by the broad expression of CDX2 (McCracken et al., 2011; Spence et al., 2011b). This work further identified that maximal and sustained CDX2 expression required the activity of both FGF and WNT signaling and that posterior specification required 4 days of exposure to FGF/WNT (Spence et al., 2011b). Together, these findings demonstrate that embryonic studies inform our approaches to direct differentiation of PSCs into posterior cell types and highlight how PSC cultures can be used to identify new developmental mechanisms regulating endoderm organogenesis.

In addition to their involvement in establishing early posterior endoderm fate, FGF, WNT, BMP, and Cdx factors play multiple roles at later stages of intestinal development including intestinal elongation, segment identity, and growth. In mice, disruption of Wnt/β-catenin signaling by deletion of the transcriptional cofactors Tcf1 and Tcf4 (Tcf1/–/–;Tcf4/–/–) resulted in a loss of Cdx2 expression and ectopic Sox2 expression at e13.5 (Gregorieff et al., 2004), a phenotype that is similar to deletion of Cdx2 in the mid-gestation embryo (Grainger et al., 2010). Deletion of Wnt5a caused shortening of both the small and large intestines as well as disruption of apical–basal polarity of the epithelium (Yamaguchi et al., 1999; Cervantes et al., 2009). BMP ligands that are expressed in the posterior mesoderm act in a signaling loop with Sonic Hedgehog from the endoderm to regulate posterior Hox13 gene expression in the hindgut of chick embryos (Roberts et al., 1995). Lastly, FGF10 signaling, mediated through FGFR2IIb, is involved in the development of several regions of the intestine and embryos with defective FGF10 signaling have cecal defects and colonic atresia (Burns et al., 2004; Sala et al., 2006). It should be possible to manipulate these pathways to regulate regional identity of HIOs, although this has not yet been reported.
WNT, and FGF pathways were known to function in endoderm posteriorization, how they act to regulate gut tube morphogenesis is poorly understood. It was known that non-canonical Wnt signaling may play a role in posterior development and intestinal morphogenesis, promoting elongation of the endodermal hindgut during development (Yamaguchi et al., 1999; Cervantes et al., 2009; Zhang et al., 2013). However, the first evidence that FGF and canonical WNT may cooperate during gut tube morphogenesis came from HIO studies. In this case, the combined activities of FGF4 and canonical WNT signaling promoted the morphogenesis of a 2-D sheet of DE into 3-D gut tube-like structures, termed gut tube spheroids (McCracken et al., 2011; Spence et al., 2011b). While the molecular basis for how FGF and WNT act to promote gut tube morphogenesis is not known, the ease of manipulating and imaging HIO cultures should allow for a better understanding of this process.

Another key event that occurs during hindgut tube formation is the assembly of a primitive mesenchyme around the gut tube epithelium. In HIO cultures, a mesenchymal layer assembles next to the gut tube epithelium during morphogenesis, although it is not known which cell types drive morphogenetic processes. The source of the mesenchyme in these cultures is a small population of mesendoderm that expands in response to FGF4. It is also possible that FGF drives morphogenesis through directed cell migration of mesoderm, as has been observed in chick, where FGF4 and 8 act as

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**Figure 1. Generation of HIOs from PSCs.**

Early stages of embryonic development guide the generation of HIOs from pluripotent stem cells into intestine. Definitive endoderm is established by the end of gastrulation (E7.5 in the mouse) and requires TGFβ molecule Nodal signaling. Differentiation of PSCs into definitive endoderm is achieved using the Nodal mimetic Activin A, which promotes differentiation into definitive endoderm as marked by Foxa2 (green) and Sox17 (red). The 2-dimensional sheet of DE folds to form a 3-dimensional primitive gut tube that is functionally subdivided along the anterior–posterior axis into a foregut, mid-gut, and hindgut (E8.5 in the mouse). Morphogenesis and posteriorization are induced in DE cultures via activation of FGF/WNT signaling. This causes the 2-D endoderm monolayers to undergo morphogenesis to form 3-D spheroids that additionally express the posterior marker CDX2. Spheroids that are grown in 3-D culture conditions for 4 weeks form HIOs that express CDX2 and have all major secretory and absorptive lineages of the fetal intestine. HIOs can be routinely split and re-plated in fresh 3-D matrigel every 2 weeks and have been passaged in this manner for up to a year.
chemoattractants and repellants to direct the posterior migration of hindgut mesoderm (Lickert & Kemler, 2002; Yang et al., 2002; Kinkel et al., 2008; Bayha et al., 2009).

After gut tube formation in vivo, there are a number of morphogenic events that result in the formation of intestinal villi with differentiated columnar epithelium (Spence et al., 2011a). The simple cuboidal epithelium of the 8.5 gut tube expands and transitions to a pseudostratified epithelium by E12.5 (Grosse et al., 2011). The epithelium transitions into a columnar type of epithelium with folds that extend into the lumen, the first stage in villus morphogenesis. Mesenchyme has been implicated in villus morphogenesis in mice, where PDGFRA-positive mesenchymal clusters influence the formation of villi through Hedgehog (Hh)-mediated signaling (Karlsson et al., 2000; Walton et al., 2012). In addition, the formation of smooth muscle layers and the resulting tension on the epithelium has been shown to be required for both early and later stages of villus morphogenesis in chickens (Shyer et al., 2013).

When grown in a three-dimensional matrix, PSC derived hindgut spheroids transition through very similar stages of intestinal morphogenesis in vitro resulting in the formation of human intestinal organoids (HIOs) (Fig 1). Similar to an e9 gut tube, day 0 spheroids start as a cuboidal epithelium surrounded by a primitive mesenchyme. However, after 14 days of culture, HIOs form a pseudostratified epithelium that resembles a E12.5 mouse intestine. By 28 days, HIOs contain columnar epithelium with protrusions into the organoid lumen, similar to early villus-like structures. As the epithelium transitions through these stages, the mesenchyme also differentiates into layers containing smooth muscle cells, subepithelial fibroblasts, and fibroblasts (Spence et al., 2011b). As with other organoid systems (reviewed in Lancaster and Knoblich, 2014), there appears to be some level of ‘self assembly’ involved in the formation of HIOs. This suggests that communication between the epithelium and the mesenchyme during HIO development may resemble the signaling that occurs during gut development in vivo. Additionally, these signals are still needed in adult intestinal organoid systems and the communication has been shown to influence the epithelial ISC and their niche in these culture conditions (Ootani et al., 2009). Therefore, these organoids represent a new model to study human intestinal epithelial morphogenesis, stem cell differentiation, and cell–cell interactions.

Differentiation and function of intestinal subtypes

The major differentiated cell types in the intestine can be detected by e16.5 in the mouse, just after villus morphogenesis. These include the absorptive enterocytes, and two secretory cell types: goblet cells and enteroendocrine cells (Fig 3). Paneth and tuft cells arise after birth along with the formation of the crypts of Lieberkuhn and the establishment of adult ISCs. Absorptive enterocytes (small intestine) and colonocytes (large intestine) (Cheng & Leblond, 1974) function to transport peptides, ions, water, sugars, lipids, B12, and reabsorb bile acids. These cells contain microvilli on their apical surface to increase absorptive surface area and act as a protective brush border from the external environment (Mooseker, 1985). Though Notch signaling has been known to be the regulator between absorptive and secretory fates (Fre et al., 2005; Stanger et al., 2005; Zecchini et al., 2005), little is actually known about the specific genes required for enterocyte differentiation. Rather, the presence of Notch allows inhibition of Atoh1, a pro-secretory transcription factor, and loss of the Notch effector Hes1 promotes an enterocyte fate (Jensen et al., 2000; Yang et al., 2001; Shroyer et al., 2007; Noah et al., 2011).

In the adult, Atoh1 is required for all secretory lineages: goblet cells, enteroendocrine cells (EECs), Paneth cells, and tuft cells. Downstream of Atoh1, it has been suggested that goblet and Paneth may share a common progenitor given their dependence on Gfi1, Sdpf, and Lkb1/Stk11 (reviewed in Noah et al., 2011). Goblet cells function to secrete mucins in order to protect the epithelium from damaging agents such as acidity and microbes in the lumen. Though these cells are present in both the embryonic and adult intestine, goblet cells may not differentiate the same way, as Klf4 has been shown to be required for embryonic goblet cell fate but not for those differentiated postnatally (Katz et al., 2002; Pellegrinet et al., 2011). Paneth cells develop postnataally in the mouse following crypt formation, but are present in humans by the first trimester (Underwood, 2012). Secreting antimicrobial peptides, Paneth cells regulate innate intestinal immunity, protecting the ISCs from pathogens, and shaping the gut microbiome. Wnt/beta-catenin signaling and its downstream target Sox9 are essential for the specification of Paneth cells (Bastide et al., 2007; Mori-Akiyama et al., 2007). Paneth cell dysfunction is associated with progression of necrotizing enterocolitis (NEC) in premature infants, though our knowledge is limited due to a lack of proper disease models (reviewed in Underwood, 2012). Lastly, markers such as Gfi1b have identified tuft cells; however, the development and function of these cells are poorly understood. There has been some speculation that tuft cells may help neutralize the acidic chyme in the duodenum and function in chemoreception and inflammation (reviewed in Noah et al., 2011; Gerbe et al., 2012).

Enteroendocrine cells (EECs) occupy approximately 1% of the total epithelium in the intestine but secrete upwards of 15 different types of hormones. These hormones have a variety of roles that affect metabolism, the digestive process, satiety, hunger, and glucose homeostasis. The endocrine role of hormones in regulating glucose metabolism and satiety is well studied (reviewed in Strader & Woods, 2005; Deacon & Ahrén, 2011; Crespo et al., 2009). In both mice and humans, specification of all EECs requires the basic helix-loop-helix transcription factor Neurogenin3 (NEUROG3) (Jenny et al., 2002; Wang et al., 2006a; López-Díaz et al., 2007; Pinney et al., 2011; Rubio-Cabezas et al., 2011). Loss of NEUROG3 leads to a loss of all endocrine cells in the intestine and a subsequent failure in the absorption of nutrients. Mouse studies have demonstrated that the transcription factor Neurod1 is downstream of Neurog3 and is required for the development of CCK and secretin cells (Naya et al., 1997; Rindi et al., 1999) and Arx is required for proximal subtypes CCK, GIP, and secretin, and distal subtypes PYY, GLP-1, and neurotensin (Beucher et al., 2012). Other TFs such as Pdx1, Nkx2.2, Pax4, and Pax6 have been shown to influence the differentiation of EECs (Larsson et al., 1998; Desai et al., 2008; Chen et al., 2009; Wang et al., 2009). Interestingly, deletion of Foxo1 from the small intestinal epithelium caused EECs to express and secrete insulin (Talchai et al., 2012), suggesting that intestinal and pancreatic endocrine hormones utilize similar transcriptional machinery. Moreover, ectopic expression of MafA, Pdx1, and Ngn3 within intestinal cell crypts led to the formation of ‘neo-islets’ in the intestine (Chen et al., 2014). These papers also demonstrate that insulin-expressing cells in the
gut restore normal glucose levels in diabetic mice, suggesting that converted EECs may be used as a diabetes therapy in the future.

Using HIOs to study development of intestinal cell types

The columnar epithelium in HIOs contain all the main cell types of the human fetal small intestine including enterocytes, goblet cells, Paneth cells, enteroendocrine cells, and a proliferating progenitor zone (Spence et al., 2011b). HIO enterocytes are polarized, have a brush boarder, and have dipeptide transport activity resulting in absorption of dipeptides that are injected into the organoid lumen. Goblet cells actively secrete mucus into the lumen of the organoid, and EECs are capable of basal secretion of hormones (unpublished observation). HIOs contain proliferating progenitor cells that express SOX9, KLF5, ASCL2, and LGR5, which are localized at the base of villus-like structures. Moreover, the differentiated cell types in HIOs are present in similar proportions as their in vivo counterparts (Spence et al., 2011b). The fact that HIOs are functionally and architecturally similar to human fetal intestine, in combination with their ability to be passaged long term (Spence et al., 2011b; Talchai et al., 2012), makes them an excellent model to study human intestinal development, as well as neonatal physiology and disease. One example is seen in Spence et al., where the authors modeled loss of EECs that is caused by mutations in NEUROG3 (Wang et al., 2006a; Pinney et al., 2011; Rubio-Cabezás et al., 2011). Knocking down of NEUROG3 in HIOs similarly resulted in loss of EECs; moreover, ectopic expression of NEUROG3 caused precocious differentiation of EECs. Because rodents lack some of the hormone subtypes, such as motilin that are found in humans, HIOs are an ideal model to study specification of endocrine cell lineages. Two recent examples used genetic manipulation of HIOs to convert human EECs into insulin-expressing cells (Talchai et al., 2012; Chen et al., 2014). Additionally, using CRISPR/Cas9 system, McGrath et al., have mutated the NEUROG3 locus in PSCs, a tool that could provide means to model or correct human mutations causing rare intestinal diseases. Moreover, induced pluripotent stem cell lines from patients with genetic forms of intestinal diseases such as cystic fibrosis and intractable diarrhea in infancy (enteric endocrinosis) could be used to generate HIOs to model the diseased phenotype, providing a means to understand the biology behind EECs and nutrient absorption as well as how to go about treating these patients.

Much is known about adult ISC maintenance and their ability to differentiate into all of the cell types of the adult intestinal epithelium. For example, Wnt/β-catenin signaling components are essential for maintenance of the ISCs in the crypt, whereas BMP signaling is involved in differentiation of progenitors. Wnt signaling has also been implicated in the formation of ISCs from a very early intestinal progenitor population (Fordham et al., 2013; Mustata et al., 2013); however, establishment of the niche and the exact mechanisms of action have yet to be elucidated. As the first in vitro model of human intestinal development, HIOs are a potentially valuable tool to identify mechanisms by which fetal intestinal progenitors give rise to differentiated cells types as well as intestinal stem cells. Manipulation of signaling pathways with small molecules, genetic manipulation of stem cell factors, and real-time imaging using cell-specific reporter lines makes HIOs a powerful new tool to study ISC development.

Maturation of PSC-derived HIOs in vivo

Immaturity is a common feature of human PSC-derived cells and tissues and has been reported for liver hepatocytes, pancreatic beta cells, and cardiomyocytes (Mummery et al., 2002; Shirahashi et al., 2004; D’Amour et al., 2006). Despite the remarkable architectural and cellular similarities to fetal intestine, HIOs do not have molecular and structural features of the adult intestine. For example, the villus-like structures that form are short and fetal-like, there are no crypts and there is no evidence for the presence of functional ISCs. Possible reasons for an immature phenotype could be that 4-week-old HIOs have not had time to mature. HIOs can be cut into smaller pieces and passaged in vitro for up to a year, and older HIOs acquire a somewhat more mature phenotype (Spence et al., 2011b; Talchai et al., 2012). Another cause of immaturity could be missing cell types such as vascular cells or systemic factors that could only be found in vivo. In vitro, the maximal size of an HIO is about 2 mm, which is likely due to limitations of oxygen and nutrient diffusion.

To overcome the absence of a functional vasculature system in vitro, a recent study described the growth of HIOs following engraftment under the kidney capsule in mice (Watson et al., 2014). Engrafted HIOs became vascularized by the host endothelial cells and after 6 weeks grew into intestinal tissue ranging from 1 to 2 cm in size. The resulting tissue had mature villi with a full complement of highly differentiated adult cell types and maintained regional intestinal identity in the host environment (Watson et al., 2014). HIOs matured in vivo also had bona fide crypts that contained Paneth cells and cells expressing ISC markers LGR5 and ASCL2. Moreover, isolated crypts had ISC activity in that they gave rise to expandable enteroids in ISC growth conditions (Sato et al., 2009). Lastly, HIOs grown under the kidney capsule were able to proliferate in response to small bowel resection (SBR), resulting in expansion of crypts and lengthening of villi, establishing this as a model to identify humoral factors regulating intestinal adaptation following SBR. These data suggest that HIOs engrafted in vivo can be used to study intestinal development and maturation, for example, the processes involved in specification of ISCs and the niche (Fig 2).

Tissue complexity of the intestine

In addition to the diversity of epithelial cell types that formed in HIOs in vivo, the HIO mesenchyme differentiated into three stratified layers of smooth muscle, a smooth muscle adjacent to the mucosa, and two additional layers of smooth muscle, as well as myofibroblasts and stromal fibroblasts (Watson et al., 2014). Moreover, HIOs grown in vitro develop a vascular system that is largely derived from the host. However, there are other cell and tissue types missing from HIOs that are normally found in the intestine, including mesenteric lymph nodes called Peyer’s patches, an enteric nervous system, epithelial M cells, and Brunner’s glands. In addition, HIOs lack microbes that are normally found in the intestine (microbiome), which play essential roles in digestion and metabolism. Therefore, for fully functional intestinal tissues to be derived from PSCs, it may be necessary to incorporate these missing components (Fig 3). Below we will discuss the embryonic development of some of these cell types and how HIOs could be engineered to contain additional complexity.
The main role of the enteric nervous system (ENS) of the intestine is to coordinate peristalsis for unidirectional movement of lumen contents. There are more than 100 million neurons in the human ENS (for review, see Goldstein et al., 2013), which includes motor, sensory, and interneurons. Developmentally, the enteric nervous system arises from a multi-potent population of cells called neural crest cells (NCCs) that migrate into the mesenchyme of the foregut (Lake & Heuckeroth, 2013). From here, they migrate rostrally and proliferate to fully colonize the gut by e14.5 in the mouse and week seven of human gestation. Migration and proliferation is driven in part by signals from the mesenchyme, including GDNF, Netrin, and BMP signaling that act via receptors on NCCs (Cacalano et al., 1998; Wu et al., 1999; Maniê et al., 2001; Zhu et al., 2004). Enteric NCCs (ENCCs) will give rise to up to 18 different subtypes of neurons and glia that will form the outer myenteric plexus and the inner submucosal plexus. These plexuses are embedded within submucosal smooth muscle layers and the circular and longitudinal layers and coordinate with the interstitial cells of Cajal (ICCs) that serve as a pacemaker for peristalsis (for review on ICCs see Huizinga & Chen, 2014). In addition, the ENS and the gut epithelium are in constant communication to maintain gut barrier function, control endocrine secretions, and regulate blood flow. For example, gut hormones such as 5’HT directly stimulate ENS neurons to stimulate peristaltic movements, secretion, and vasodilation of the gut (reviewed in Mawe & Hoffman, 2013). Secretin and CCK have been shown to stimulate sensory neurons, which then act on the pancreas to release HCO$_3$ into the duodenum as well (Nathan & Liddle, 2002; Li, 2007). Ghrelin also acts through a neuronal pathway, in order to stimulate the hunger response in basal conditions (Andrews, 2011) via a brain–gut axis.

The ENS also regulates the barrier function of the epithelium. The intestinal epithelial barrier (IEB) is composed of apically located tight junctions, as well as adherent junctions that confer mechanical strength, regulate permeability, and anchor the epithelium. Through regulation of protein subunits of epithelial junctions, the ENS controls intestinal paracellular and transcellular permeability changes that occur during postnatal development and injury (reviewed in Neunlist et al., 2013). Neuromediators such as acetylcholine and substance P increase permeability and cell proliferation, whereas VIP decreases it (Goode et al., 2003; Toumi et al., 2003; Cheng et al., 2008). In addition, glial cells secret S-nitrosoglutathione (GSNO) in response to bacterial infection, resulting in down-regulation of CDCA2 and a decrease in cytoskeletal recruitment (Flamant et al., 2011). This increases the barrier resistance to bacteria such as *Shigella flexneri*. Lastly, in cases of intestinal injury, enteric glial cells secrete proEGF, which acts on enterocytes to increase

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**Figure 2. Unique advantages and limitations of current intestinal organoid systems.**

HIOs grown in vitro are similar to fetal intestine and thus are uniquely suited to study human intestinal development using both genetic and pharmacologic manipulation of genes and signaling pathways. HIOs also contain mesenchyme allowing for studies of epithelial–mesenchymal interactions. HIOs grown in vivo contain intestinal stem cells, crypts, villi, and differentiated smooth muscle layers and can be used for functional studies of human intestine. Primary cultures of human intestinal crypts (called enteroids (ENOs) or adult intestinal organoids) have more mature characteristics and can be used to study intestinal stem cell biology. Lastly, direct analysis of patient tissues provides an important snap shot of normal and pathological states of patient tissue; there is no opportunity for experimental manipulation. Both in vitro systems share many advantages in studying intestinal biology and disease modeling.

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**ADVANTAGES**

- Intestinal development
- Epithelial–mesenchymal interactions
- Mesenchymal development

**LIMITATIONS**

- Lacks adult function
- Lacks ENS, vasculature, immune cells
- Derivation requires PSC skill sets

**ADVANTAGES**

- Study cell functionality
- Epithelial maturation
- ICCs and the niche

**LIMITATIONS**

- More adult function
- ISC studies
- Postnatal diseases

**ADVANTAGES**

- Full tissue complexity
- Full disease pathology

**LIMITATIONS**

- Limited amount of tissue
- Tissue quality variable
- No genetic manipulation

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**Human intestine**

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**In vitro HIO**

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**In vivo HIO**

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**Ex vivo ENO**
focal adhesion kinase (FAK) (Van Landeghem et al., 2011). FAK has major roles in intestinal cell motility, which allows cell spreading over the damaged area and increased mucosal healing.

A close association of the vasculature with the gut epithelium is essential for proper absorption and systemic transport of nutrients and hormones. The intestinal vasculature consists of 2 large mesenteric arteries that branch into arterioles, which span from the muscularis layer down to single villi. In addition, a separate arteriole branch exits the villi and submucosal layer in the opposite direction, to transport absorbed nutrient and intestinal hormones throughout the gut.

Figure 3. Complexity of the intestine.
The intestine is an assembly of multiple cell types from all 3 germ layers. The endoderm gives rise to the epithelium of the intestine (insert). This region is known as the mucosa. This includes cell subtypes such as enterocytes (yellow), enteroendocrine cells (blue), Paneth cells (pink), and goblet cells (green). Capillaries and blood vasculature as well as myofibroblasts and Peyer’s patches/M cells control transport of nutrients, epithelial integrity, and immune responses, respectively. The epithelium is circled by three smooth muscle layers that are arranged in alternating longitudinal–circular–longitudinal orientations. Embedded within the muscle is the enteric neural system (ENS), the submucosal plexus and the myenteric plexus. While HIOs contain most of these epithelial and mesodermal cell types, other cell types such as neurons will need to be incorporated in order to more accurately mimic in vivo development and adult function. In addition, microbial components can be injected into HIOs to study immunity, gut barrier functions, and metabolism by the microbiome.
The intestinal microflora

Intestinal function depends on an extensive array of commensal and symbiotic bacteria that mediate a number of positive symbiotic host–bacterial interactions. At birth, the intestine is sterile but over time becomes populated with over 1,000 different species of microbes (Lozupone et al., 2012) that have very important roles in digestion, stimulating the immune system, acting in various metabolic processes, and providing a barrier against disease-causing pathogens. It is estimated that the number of bacteria in the gut outnumbers the cells of our body by 10:1 (Sekirov et al., 2010). The types of microbes that colonize the gut in postnatal stages of development are highly variable (reviewed in Putignani et al., 2010) and are influenced by antibiotic exposure during pregnancy, bacteria in the amniotic fluid, hygiene, weight gain during pregnancy, and route of birth (vaginal versus cesarean). Dysbiosis of bacterial/epithelial interactions can correlate with the development of intestinal disorders such as irritable bowel syndrome (IBS) (Li et al., 2014a; Wang, 2014), and obesity and diabetes (Turnbaugh et al., 2006; De La Serre et al., 2010; Han & Lin, 2014). Introduction of bacteria into the lumen of HIOs would allow for methodical screening for how patient microbiomes can impact the host epithelial maturation and function.

Engineering additional functionality into human intestinal organoids

HIOs are a highly complex organoid system containing over 10 different cell types. However, many of the cell types described above are lacking, and the generation of fully functional intestinal tissue from PSCs will ultimately require incorporation of these cells. For example, though HIOs contain mesenchymal cell types including smooth muscle, fibroblasts, and myofibroblasts, they do not have HIO-derived vascular endothelial cells and it is not clear whether they contain interstitial cells of Cajal. Moreover, sub-mucosal populations of epithelium such as Brunner’s glands have not been identified in HIOs, and it is not clear whether the epithelium is competent to generate these specific cell populations. One of the most critical components lacking in HIOs is the enteric nervous system/ENS, and we will use this as an example of how one might engineer additional complexity into HIOs. The ENS is an ectodermal cell type deriving from neural crest cells/NCCs. Given that NCCs are not present in HIO cultures, the only way to generate HIOs with an ENS would be to mechanically incorporate NCCs into HIOs at some stage during their differentiation. First, one would have to generate NCCs in vitro and there are established protocols for the generation of cranial NCCs from PSCs (Chambers et al., 2009; Menendez et al., 2011, 2013; Kreitzer et al., 2013). However, vagal/sacral NCCs, which are the source of the ENS, have not yet been generated in vitro. Therefore, cranial NCCs would have to be posteriorized to adopt a more vagal/sacral NCC fate and then incorporated into the developing HIOs. As with all PSC differentiation protocols, the best approach has always been to follow normal developmental timing. Given that NCCs migrate into the gut tube mesenchyme shortly after gut tube formation, incorporating NCCs shortly after gut tube/spheroid formation would be the most biologically logical choice. If NCCs could be incorporated into HIOs, it would allow for in vitro analysis of ENS migration, proliferation, and differentiation of different neural cell types and assembly into the two neural plexuses. Little is known about how NCCs interact with the mesenchymal and endodermal cells during intestinal development and how the mature ENS modulates epithelial and immune function. Importantly, building additional functionality into HIOs would allow for studies of complex diseases like Hirschsprung’s disease, irritable bowel syndrome, cancer, and infection via bacterial pathogens, as discusses below.

Current and future uses of HIOs for intestinal disease research

Intestinal diseases are caused by parasitic, genetic, environmental, and sometimes a combination of these factors and affect
60–70 million people in the USA. In the following section, we address some of the major disorders of the intestine and how organoids are being used to uncover new disease mechanisms (Fig 4).

**Infectious diseases**

There are two examples where HIOs have been previously used to study enteric pathogens. In one example, the microorganism *C. difficile* was injected into HIOs and resulted in epithelial barrier dysfunction, similar to *in vivo* infections (Leslie et al., 2014). A second study involved rotavirus, for which current cell culture models were inadequate because they did not support viral replication. In contrast, HIOs supported the rotovirus life cycle and now represent a relevant model system to study the pathology of this gastrointestinal viral infection (Finkbeiner et al., 2012). Moreover, the use of PSC reporter lines that mark specific subpopulations of cells, such as a recently reported LGR5-GFP line (McCracken et al., 2014; Watson et al., 2014), would allow for identification of which cell types are targeted by these various pathogenic agents. There are, however, many enteric pathogens for which no good human model exists. *Gardia duodenalis* (*G. duodenalis*) is a protozoan parasite that inhabits the upper small intestine and causes the disease human giardiasis. The parasite spreads via a fecal–oral route, most often from contaminated food or water. During infection, the parasite induces apoptosis in villus epithelial cells.

![Figure 4. Using HIOs to study intestinal development and model diseases.](image)

The *in vitro* culture of human intestinal tissues provides new opportunities to study cancer, infection, and genetic diseases. This is essential since certain human diseases, such as colon cancer, and enteric pathogens, such as *C. cayetanensis*, are not effectively studied in other model systems. This makes studying human intestinal disease very difficult. In addition, HIOs could be used as a rapid primary screen for drug absorption and GI toxicity, the most common off-target effect of new drugs.
hyperplasia of crypts, as well as intestinal barrier dysfunction (reviewed in Halliez & Buret, 2013). So little is known about the pathophysiology of this parasite that it has been recently put on the World Health Organization’s Neglected Disease Initiative (World Health Organization, 2004; Savioli et al, 2006). *Cyclospora cayetanensis* (*C. cayetanensis*) is another food- or water-borne parasite for which the only known host is the human small intestinal epithelium. Considered an emerging pathogen, *C. cayetanensis* infection and outbreaks are quite difficult to diagnose (Eberhard et al, 1997; Mansfield & Gajadhar, 2004). For pathogens such as these, human intestinal organoid systems are the only known option for understanding the biology of infection.

**Genetic diseases**

There are several common genetic diseases that affect intestinal epithelial function. For example, autosomal recessive mutations in the cystic fibrosis trans-membrane regulator (*CFTR*) gene lead to decreased Cl⁻ ion movement through epithelial cells (Riordan et al, 1989), hyperabsorption of fluids, and subsequent dehydration of epithelial cells. While cystic fibrosis (CF) has pathology in many epithelial tissues, in the intestine it affects the motility of luminal contents, causes bacterial overgrowth, increases inflammation and ulceration, as well as leads to the accumulation of mucus. Aberrant secretion and altered mucus content often leads to intestinal blockage and malabsorptive phenotypes. Current small molecule therapies for CF are only modestly effective, and human intestinal organoids represent a new system for high-throughput, functional screens for new therapeutic compounds. For example, intestinal organoids derived from surgical biopsies of CF patients had a measurable disease phenotype that was corrected with an existing CF drug (Dekkers et al, 2013). Several groups have derived iPSC lines from CF patients (Mou et al, 2012), and HIOs from iPSC lines with specific CF mutations could uncover developmental reasons for why different CF mutations associate with more severe intestinal disease.

Hirschsprung’s disease (HSCR) is characterized by absence or paucity of neurons in the intestine and occurs in about 1:5,000 live births (McKeown et al, 2013). Depending on the severely, this can cause severe megacolon, chronic constipation, and a distended abdomen, requiring surgical resection of the aganglionic region. Several mutations have been shown to affect NCC migration, proliferation, and innervation including GDNF-GFRA1-Ret signaling (reviewed in McKeown et al, 2013), *Endothelin 3*, and *Sox10* (Kapur, 1999; Southard-Smith et al, 1998; Cantrell et al, 2004). Single allele mutation of *RET* in humans can cause the disease; however, this is not the case in mouse (Schuchardt et al, 1994; Gianino et al, 2003). An HIO-based approach might be an effective way to study the molecular basis of HSCR, but would require the incorporation of the neural crest-derived ENS progenitor cells. In support of this concept, adult neural progenitors from human or mouse healthy intestinal tissue have been incorporated into aganglionic fetal intestinal explants in culture and *in vivo* mouse models of HSCR (Natarajan et al, 1999; Bondurand et al, 2003; Dupin & Sommer, 2012). When incorporated, these neural progenitors differentiated and reduced the severity of the HSCR phenotype, yet formation of a complete neural plexus was not observed. A similar approach could be used with patient-specific NCC-derived neural progenitors and HI.Os.

There are several additional multifactorial diseases that affect the intestine and colon. Inflammatory bowel disease (IBD), which is chronic inflammatory condition, can affect the small intestine, as with ulcerative colitis (UC), or large intestines, as with Crohn’s disease (CD). Immunosuppression is the primary therapy, yet it is not known what triggers the abnormal immune response. Studies suggest that alterations in the microbiome may play a role in IBD (Li et al, 2014a; Wang, 2014). The ease of manipulating HIOs makes them amenable for IBD studies. For example, it has been shown that organoids can be injected with bacteria and this can be used to study early disease inductive events (McCracken et al, 2014). Moreover, it should be possible to study inflammatory responses by incorporating macrophages from healthy and IBD patients into HIO cultures. It may also be possible to repair damage caused by IBD using human colonic stem cells. In mice, it has been shown that engrafment of Lgr5⁺-derived colonic epithelium improves acute colitis and resulted in repopulation of damaged colonic tissue (Yui et al, 2012). Organoids derived from adult mouse intestine have been used recently to model colon cancer (Li et al, 2014b). While it is not clear whether HIOs, which are fetal in nature, could be used to model intestinal cancers, HIO tissues that are matured *in vivo* have adult characteristics and could be used to induce oncogenic mutations as was done in mouse (Li et al, 2014b).

**Concluding remarks**

The intestine is a remarkable organ that incorporates cell and tissue types from all three embryonic germ layers to carry out its absorptive, secretory, endocrine, barrier, and mechanical functions. Disruption of any of these myriad of intestinal functions can result in disease, underlying the need for human model systems to study various pathologies. The complexity of this organ has made it difficult to derive intestine from PSCs using traditional 2-D approaches, thus necessitating the development of 3-D, organoid-based systems. While the current HIO system has provided new opportunities for developmental and disease research, tissue engineering additional complexity into HIOs will allow for increased functionality and expanded utility.

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**Conflict of interest**

The authors declare that they have no conflict of interest.

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The EMBO Journal 13


