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Recent advances in deriving human endodermal tissues from pluripotent stem cells

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The utilization of directed differentiation of human pluripotent stem cells to generate human tissues is quickly evolving. Here we review recent advances in the derivation and applications of human endodermal tissues, including the esophagus, lung, pancreas, liver, stomach, small intestine, and colon. Improvements in tissue transcriptional and functional maturation, multicellular complexity, and scalability allow better development and disease modeling, large-scale drug and toxicity screening, and potentially cell therapeutic applications.

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Over the past decade, the generation and use of human tissues derived from directed differentiation of human pluripotent stem cells (hPSCs) have advanced at an extraordinary pace with many seminal discoveries made every year. Guided by development, we now possess the ability to generate a vast number of cell and tissue types originating from all three embryonic germ layers. For example, hPSC-derived definitive endoderm is capable to differentiate into gastrointestinal organoids by controlling the signaling required for embryonic anterior–posterior and dorsal–ventral patterning (Figure 1). While comprehensive reviews of organoid technology and applications have been published [1–5], this review will focus on recent advances in generating, maturing, and using human endodermal-derived tissues, including the gastrointestinal tract, lungs, pancreas, and liver.

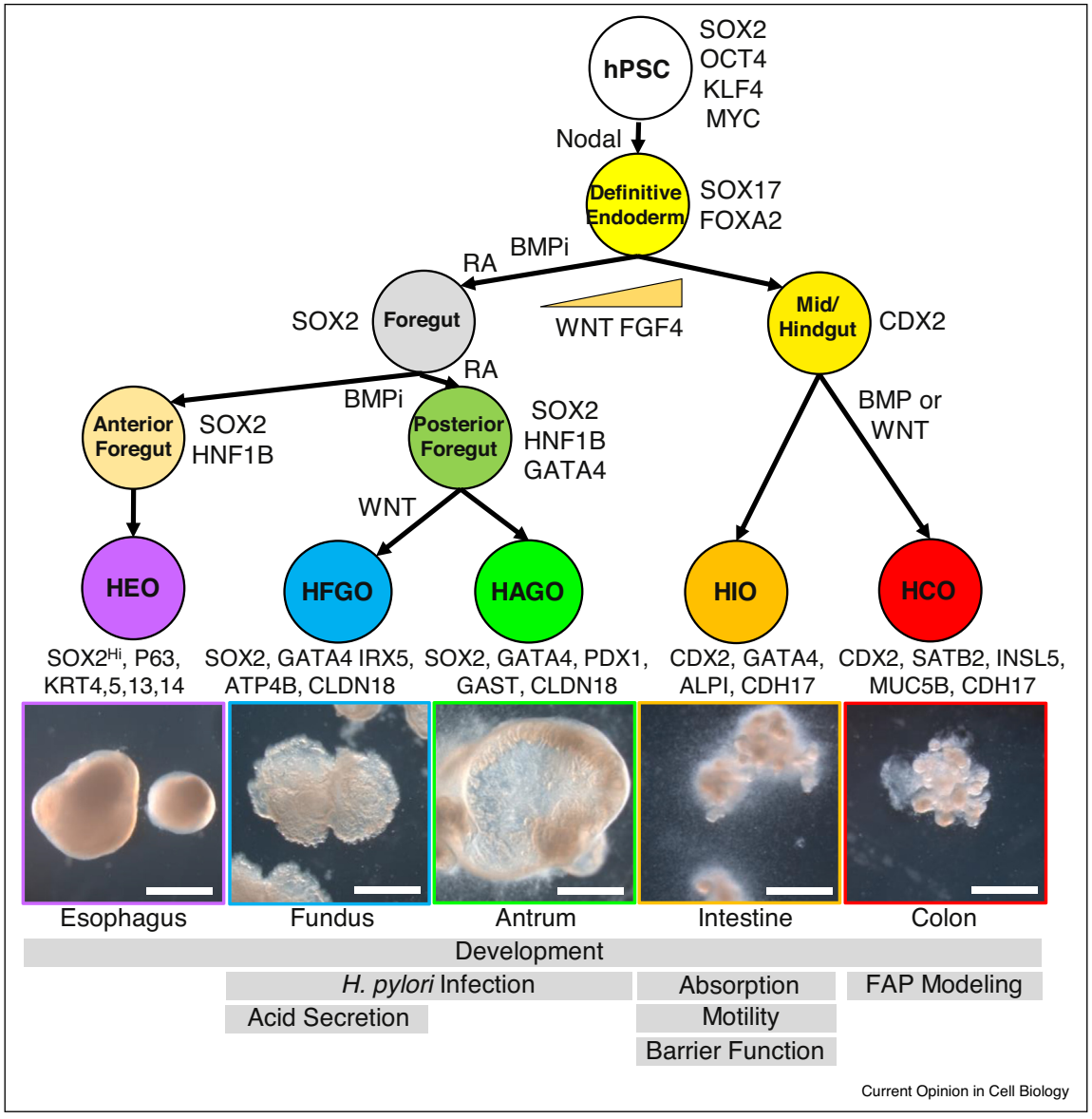
Esophagus — new frontier of human esophageal research

The direct differentiation of hPSC into human esophageal organoids (HEOs) was recently reported [6••,7•]. By mirroring normal development of the esophagus, manipulation of the TGFβ/BMP, FGF, and retinoic acid signaling pathways directed the differentiation of definitive endoderm into $SOX2^{+}P63^{+}$ dorsal anterior foregut and then HEOs (Figure 1). These HEOs had a remarkable level of transcriptional maturity when compared to human esophagus and contained basal and suprabasal cells that formed a stratified squamous epithelium, particularly when grown on an organotypic raft culture (Figure 2a) [6••,7•]. HEOs were used as a model to study the molecular pathways regulating normal esophageal development and pathways that cause esophageal birth defects. For example, BMP and Jagged2-NOTCH signaling were shown to be required for suprabasal and keratin differentiation [7•]. Mutations in SOX2 cause esophageal atresia in humans and using HEOs as a model system, Trisno *et al.* showed that defects in esophageal development caused by SOX2 loss may be due to ectopic activation of WNT signaling [6••]. While both of these new HEO systems are well-suited to study esophageal epithelium, they lack smooth muscle, enteric nerves and immune cells, which are critical for normal esophageal function and for modeling complex diseases like trachea-esophageal fistulas, motility defects, eosinophilic esophagitis, and Barrett's metaplasia.

Lung — patterning airway and alveolar organoids

The first report of hPSC-derived respiratory organoids used a directed differentiation approach to generate both proximal and distal lung tissues [8,9]. Recently, numerous additional methods to differentiate hPSCs into regional-specific lung tissues have been reported (summarized in Ref. [9]). During human lung development, NKX2-1 ventral foregut endoderm and fetal buds tip progenitors have the potential to differentiate into proximal airway and distal alveolar epithelium (Figure 2b) [8–14,15••,16]. A number of lung differentiation protocols use cell sorting enrichment of NKX2.1-expressing progenitors for long-term cultures [13,15••,16]. Single cell RNA-sequencing (scRNA-Seq) of NKX2-1 progenitors identified pathways, including WNT, that are associated with differentiation of $SFTPC^{+}$ alveolar epithelial type 2 (AT2) cells [13,14]. On the basis of this information, activation of canonical WNT signaling in NKX2-1 progenitors or bud tip progenitors was able to induce rare SFTPC cells [9–11,13,14,15••,16].

Figure 1

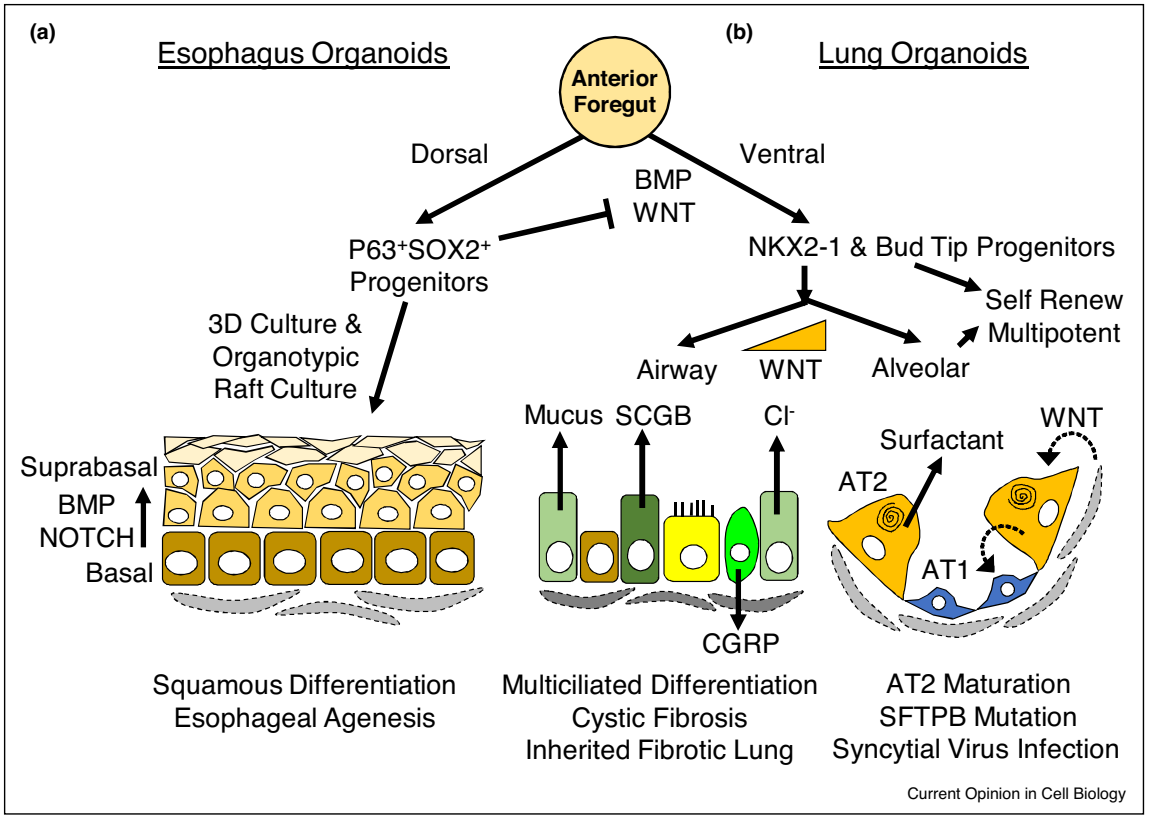


Directed differentiation of gastrointestinal organoids.
Schematic of directed differentiation of esophageal (HEO) [6••,7•], fundic (HFGO) [41,42], antral (HAGO) [39,42], intestinal (HIO) [43], and colonic organoids (HCO) [50••,51], which can be used to model tissue development, function, and disease. Representative images of gastrointestinal organoids five weeks following hPSCs (HEO are eight weeks). Scale bar represents 1 mm.

These AT2 cells could be purified, serially passaged, grown at an air–liquid interface, and differentiate into AT1 cells [9,10,14,15••], consistent with a reported subset of AT2 cells that act as a self-renewing multipotent alveolar progenitor [17,18]. HPSC-derived AT2 cells contained surfactant processing lamellar bodies [9–11,14,15••], which could functionally process proSFTPB into its mature isoform, secrete surfactant, and respond to inflammatory signaling [15••]. While WNT signaling is required for the formation of NKX2-1 progenitors and distal lung patterning [13,14,15••,16], removing WNT signaling was required for differentiation into of proximal airway cell types including basal and secretory cells, and multiciliated cells [9–11,16]. As is the case in other developing organs, WNT signaling plays distinct temporal roles during lung development and maturation.

These regionally specific lung organoids provide new opportunities to model human lung organogenesis, differentiation, and disease. Airway organoids derived from cystic fibrosis patient's iPSCs showed impaired CFTR function using the forskolin-swelling assay

Figure 2



Directed differentiation of esophageal and lung organoids.
(a) P63+SOX2+ esophageal progenitors grown in organotypic raft culture formed a stratified squamous epithelium [6••,7•]. BMP and NOTCH signaling are required for suprabasal differentiation [7•]. SOX2 repressed WNT signaling required for NKX2-1 respiratory competence [6••]. **(b)** NKX2-1 progenitors and bud tip progenitors gave rise to both airway and alveolar epithelium, the specification of which is dependent on WNT signaling [8–14,15••,16]. Airway organoids had a variety of secretory cells (green), basal (brown) and multiciliated cells (yellow) [10,11,16]. These airway organoids were used to model mechanisms of multiciliated cell differentiation and fibrotic diseases, including cystic fibrosis [11,16]. Alveolar organoids contain AT2 cells (orange), which are capable of self-renewal, differentiating into AT1 cells (blue), and have functional lamellar bodies and produce surfactant, which are defective in diseases affecting alveolar epithelium [10,11,14,15••].

(Figure 2b) [16,19]. Alveolar organoids differentiated from iPSCs of patients with SFTPB mutations lacked lamellar bodies, could not process proSFTPC or produce surfactant [15••]. Lamellar body morphology is also a potential readout for drug toxicity screening [14]. Lung bud organoids, which have both airway and alveolar differentiation potential, formed highly branched structures *in vitro* [10,11] and codeveloping mesenchyme allowed for *in vivo* transplantation and maturation [8,11]. These lung bud organoids recapitulated phenotypic characteristics of respiratory syncytial virus infection and inherited fibrotic lung disease, including alveolar epithelial cell death and increased mesenchymal proliferation and ECM deposition, respectively [11].

Pancreas — making functional β cells and modeling diabetes pathogenesis

For nearly two decades, many laboratories have strived to produce functional β cells for the treatment of patients with diabetes. The directed differentiation of hPSCs into pancreatic and endocrine progenitors has been highly successful [20,21]. However, because of our limited understanding of later stages of β cell development and maturation, hPSC-derived β-like cells were not fully functional in their response to dynamic glucose levels and negative feedback regulation [5,22–24,25•,26,27•,28,29]. ScRNA-Seq performed throughout the *in vitro* β cell differentiation protocols provided valuable insight necessary to improve the differentiation of functional β cells [26,28]. During development, β cells are part of an aggregate of endocrine cells called islets. Our understanding of islet morphogenesis is still evolving [24], but it is clear that mechanical transduction from the extracellular matrix (ECM) plays a significant role. Low levels of integrin-mediated YAP signaling are critical for the specification of endocrine progenitors, as well as inhibiting proliferation to promote differentiation and functional maturation of *in vitro* derived β cells

(Figure 3a) [29,30,31••]. To mimic β cell maturation caused by compaction during islet formation, immature insulin expressing β cells were reaggregated in a similar stoichiometric ratio of human islets (Figure 3b) [25•,26]. These scalable β cells clusters were more metabolically and functionally mature, indicated by a switch to oxidative phosphorylation and their improved ability to respond to dynamic glucose challenge relatively similar to isolated islets [25•,26]. Upon transplantation, these β cells clusters were quickly vascularized, persisted for months, and could functionally response to acute glucose challenge within days after transplant [25•].

Human tissues derived from hPSC can be used to model the etiology and pathogenesis of diabetes. A toxicity screen on heterogeneous pancreatic cultures identified that the pesticide, propargite, caused dose-dependent DNA damage and necrosis specifically in β cells, due to their low glutathione levels (Figure 3b) [27•]. β cells derived from patient iPSCs with glutathione *S*-transferase variants were highly susceptible to propargite. The pathogenesis of diabetes extends beyond the pancreas, where high circulating glucose causes diabetic vasculopathy, which is not well understood. Human blood vessel organoids, composed of codeveloping endothelium and pericytes, were treated with high glucose and inflammatory cytokines to mimic diabetic microvasculature [32•]. Recapitulating diabetic patient pathologies, these organoids revealed drastically increased ECM deposition mediated by altered DLL4-NOTCH3 signaling between the endothelium and pericytes. These studies utilizing human-derived tissues for large-scale toxicology screening and elucidation of mechanisms of diabetes are prime examples of the applications of evolving organoid technology.

Liver — building the hepatic microenvironment for pharmaceutical applications

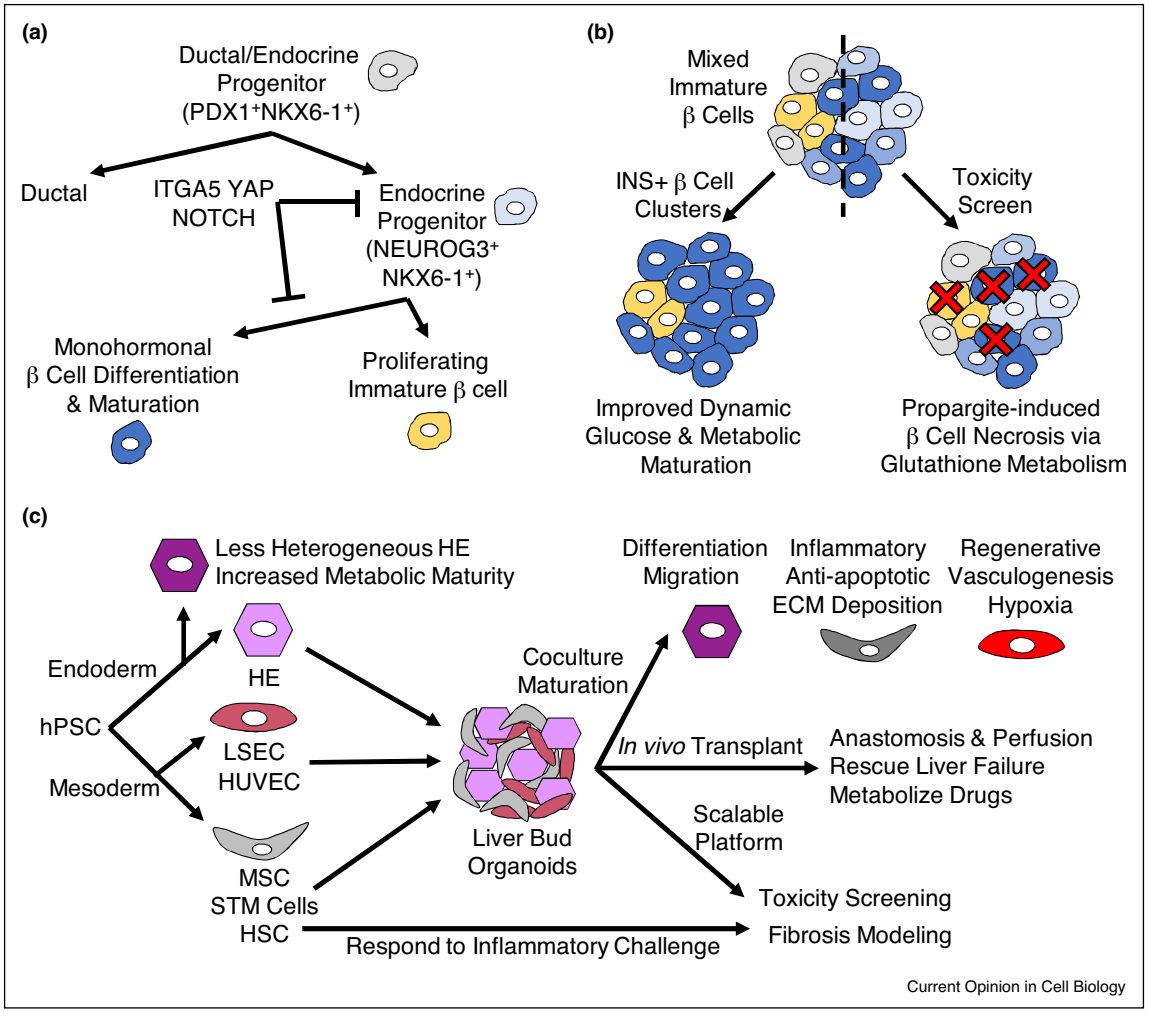
Research using liver organoids continues to utilize innovative approaches to understand the hepatic microenvironment, produce functional tissue for transplantation, and develop large-scale drug screening platforms. ScRNA-Seq revealed considerable heterogeneity during hepatic endoderm specification from definitive endoderm [33••]. By closely mirroring development, Ang *et al.* mapped out the temporal signaling required for hepatic competence to improve the differentiation of homogenous hepatic progenitors, as well as deriving more metabolically mature hepatocytes [34•]. ScRNA-Seq of 3D liver bud organoids, generated by combining hepatic endoderm, mesenchymal stem cells, and HUVECs, revealed that each cell type had distinct signs of maturation due to coculture (Figure 3c), although they all remained developmentally fetal [33••,35]. These liver bud organoids had a strong hypoxia gene signature *in vitro*, which was quickly resolved *in vivo* where the hypoxic signature likely helped promote angiogenesis within the transplant. An *in silico* receptor-ligand pairing screen identified potential multicellular crosstalk within the liver bud organoids and knockdown and high throughput imaging experiments validated pathways, including VEGFA-VEGFR2, EDN1-TIE1, and JAK3, which are partially responsible for the hepatic maturation, angiogenesis, and structural integrity, respectively.

To better model liver development, function, and fibrosis, liver-specific mesenchyme is required. Multiple groups generated self-renewing hepatic stellate cells, which produce ECM and are the fibrogenic cell in the damaged liver [36–38]. Unlike cultured primary stellate cells, hPSC-derived stellate cells are basally inactive but can functionally store Vitamin A and respond to injury stimuli by increasing proliferation, migration, and ECM [36]. When challenged with acetaminophen, cocultures of stellate cells and hepatocytes had evidence of increased fibrosis, supporting their application in toxicity screening. Liver sinusoidal endothelial cells (LSECs) can also be differentiated from hPSCs, which upon transplantation mature, self organize around hepatic endoderm, and form connections with mouse vasculature [37,38]. These hPSC-derived stellate cells and LSEC enhanced hepatic endoderm maturation more than mesenchymal stem cells and HUVECs [37]. Using hPSC-derived hepatic endoderm, LSECs, and septum transversum mesenchyme, Takabe *et al.* engineered a scalable platform to generate thousands of reproducible liver bud organoids with the ability to metabolize drugs and improved liver failure survival when transplanted into mice [38]. These recent innovations to improve hepatic organoids biological function, reproducibility and scalability bring them closer to clinical and pharmaceutical applications.

Stomach — making acid

Previously generated antral organoids lack the cells required for gastric acidification [39,40]. McCracken *et al.* identified that low WNT signaling repressed antral identity in order to specify the fundic region of the human stomach (Figure 1) [40,41]. This study established that MEK inhibition and BMP activation are sufficient for the differentiation of acid-secreting parietal cells. Acid secretion could be stimulated with histamine and blocked with proton pump inhibitors, making these fundic organoids a novel model to study gastric acid secretion [40–42]. Furthermore, while pathogenic *Helicobacter pylori* infection induced hyperproliferation in both antral and fundic organoids, only acid-secreting fundic organoids had a SHH-dependent induction of PD-L1 expression and spasmolytic polypeptide-expressing metaplasia. This illustrates the importance of both gastric organoid models to understand regional-specific therapeutic strategies [42].

Figure 3

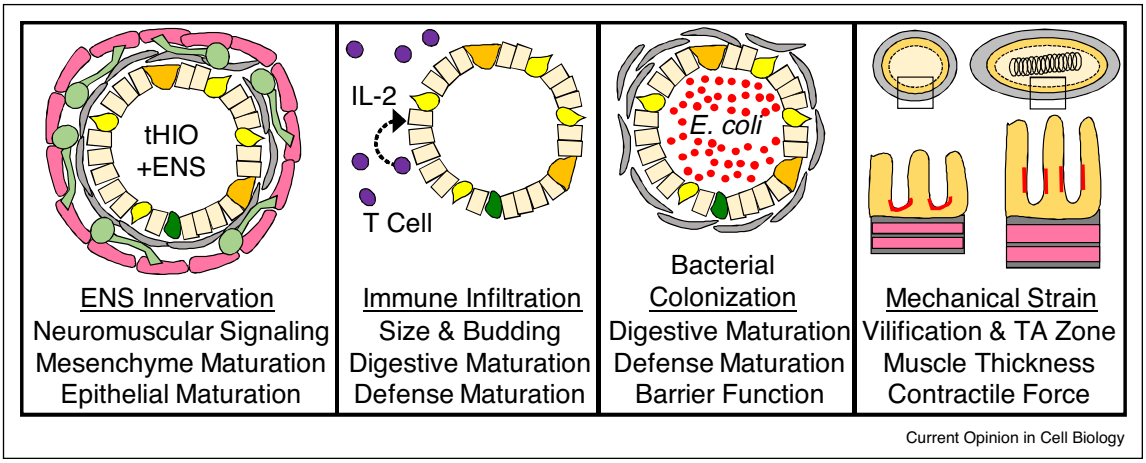


Maturation of pancreatic β cell and hepatic endoderm for pharmaceutical and clinical applications.
(a) Proliferation and differentiation of PDX1 ductal/endocrine and β cell maturation are regulated by ECM activation of ITGA5 YAP1 NOTCH signaling [29,30,31••]. **(b)** Mixed populations of immature β cells can be functionally and metabolically matured by clustering insulin β cells [26,27•] or utilized in toxicity screening to identify environmental factors that selectively kill β cells [27•]. **(c)** Differentiation of hPSCs into endodermal and mesodermal hepatic lineages, including heterogeneous and more mature hepatic endoderm (HE) [33••,34•,35], liver sinusoidal endothelial cells (LSEC) [37,38], as well as the septum transversum mesenchyme (STM) [38], mesenchymal stem cell (MSC), or functional hepatic stellate cells (HSC) [36,37]. Together these cells formed multicellular liver bud organoids, which differentially matured the individual cell types, could be transplanted to rescue rodent liver failure, and are scalable to perform large-scale toxicity screens [33••,35,36,38].

Small intestine — maturing through complexity and mechanical stimulation

Since the first generation of human intestinal organoids (HIOs) from hPSCs [43], the focus has been to enhance functionality, complexity and scale up of HIOs, with the goals of modeling intestinal physiology, disease processes, and transplantation-based therapeutics. Initial efforts to transplant HIOs into mice identified that growth *in vivo* over the span of 8–12 weeks resulted in tremendous growth and functional maturation [44,45]. Despite this, the intestinal epithelium was still not equivalent to postnatal human intestinal tissue [45] and was lacking important cell types that may promote functional development. To ascertain the importance of these additional components, enteric nerves, immune cells, microbiota, and mechanical strain, all of which play a role in intestinal maturation, have been engineered into HIOs (Figure 4). The enteric nervous system (ENS) of the gastrointestinal tract comes from neural crest cells. Incorporation of hPSC-derived neural crest cells into HIOs resulted in formation of enteric nerves and glia that formed functional neuromuscular connections with smooth muscle cells capable of peristalsis when matured *in vivo* [46]. Furthermore, incorporation of an ENS

Figure 4



Diverse mechanisms to mature intestinal organoids.
Methods to introduce complexity and maturity to HIOs. Introduction of the enteric nervous system (ENS) into the mesenchyme promoted epithelial and mesenchymal maturity and the differentiated neurons (green) formed functional neuromuscular synapses that regulated smooth muscle (pink) contraction in transplanted HIOs (tHIO) [46]. Immune cytokine IL-2 increased organoid growth and matured the digestive and defensive machinery in the epithelium [47]. Likewise, colonization by non-pathogenic *E. coli* promoted increased epithelial barrier function [45]. Mechanical strain in tHIOs promoted villi growth, a more defined TA zone (red), and smooth muscle growth increased contractile force [49].

enhanced both epithelial and mesenchymal maturation. Coculture of HIOs with T cells, their conditioned media, or IL-2 increased growth, digestive and defensive transcriptional signatures mediated through the STAT3 and mTOR pathways [47]. These immune-matured organoids had increased transporters and metabolizing enzymes, could transport glucose and pharmaceuticals across the epithelial barrier, had functional CFTR channel, and secreted more mucus and hormones. Colonization of HIOs with non-pathogenic *Escherichia coli* induced epithelial maturation, innate anti-microbial response, and angiogenic gene signatures, as well as enhanced barrier function mediated through NFκB-dependent and hypoxia-dependent mechanisms [45]. During development, mechanical forces imposed by intestinal lengthening and smooth muscle differentiation caused villus morphogenesis and the establishment of the transit-amplifying (TA) progenitor zone [48]. Similarly, introduction of longitudinal mechanical strain into the lumen of transplanted HIOs induced robust vilification, a more defined TA zone, and improved contractile force of smooth muscle [49]. These studies provide insight into intestinal maturation and can be utilized to study motility, absorption, and barrier function.

Colon — New tools to study colon development and disease

Several reports have used signaling pathways that are known to posteriorize gastrula stage embryos to posteriorize human endoderm into colonic organoids (HCOs) [50••,51]. Múnera *et al.* demonstrated that a pulse of BMP signaling immediately following mid/hindgut endoderm patterning is sufficient to initiate the posterior HOX code and a colonic-specific gene signature (Figure 1) [50••]. These HCOs expressed colonic-specific mucus and enteroendocrine cells *in vitro*, which persisted following *in vivo* maturation. Alternatively, two groups used prolonged WNT signaling to induce a posterior signature [51,52]. Differentiated HCOs from familial adenoma polyposis patient-derived iPSCs showed hallmarks of overactive WNT signaling and proliferation consistent with adenomas, which could be rescued using geneticin-induced APC mutation read-through [51]. For some applications, HCOs might represent a more physiological system than immortalized colorectal cancer cell lines as a model system to investigate human colonic agenesis, diarrhea, inflammatory bowel disease, and colon cancer.

Perspectives and future directions

Recent advances to generate human tissues via organoid-based technology have focused on recreating complex tissue microenvironments through multicellular cocultures. These approaches have opened up exciting opportunities to model human organogenesis and pediatric diseases. Currently, hPSCs-derived tissues are not equivalent to their adult tissue counterparts, and in some cases, this may limit their ability to faithfully model adult onset diseases or application as tissue therapies. However, incorporating hPSC-derived mesenchymal, neural, endothelial, and hematopoietic lineages [53–56] is allowing the field to generate more fully functional organoids. The ability to selectively add and remove particular cell types is allowing for mechanistic studies of cell–cell communication that maintains tissue homeostasis or drives disease pathogenesis when disrupted.

Efforts to study organ–organ interactions are being made possible through interconnected microfluidic devices. Moving forward, improvements in organoid reproducibility, scalability, and functional readouts for large-scale drug and toxicity screens will be a priority. Screening compounds on representative libraries of human organoids derived from diverse populations will provide invaluable insight into toxicity and potentially streamline drug approval for patients. Finally, organoids from patient-derived iPSCs will provide new diagnostic tools to better identity and understand patient-specific pathologies and even new resources to replace damaged tissues.

Conflict of interest statement

Nothing declared.

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- •• of outstanding interest

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