In vitro models of intestinal epithelium: Toward bioengineered systems

Journal of Tissue Engineering
Volume 12: 1–16
© The Author(s) 2021
Article reuse guidelines:
sagepub.com/journals-permissions
DOI: 10.1177/2041731420985202
journals.sagepub.com/home/tej

Justine Creff[1,2], Laurent Malaquin[2,*] and Arnaud Besson[1,*]

Abstract

The intestinal epithelium, the fastest renewing tissue in human, is a complex tissue hosting multiple cell types with a dynamic and multiparametric microenvironment, making it particularly challenging to recreate *in vitro*. Convergence of recent advances in cellular biology and microfabrication technologies have led to the development of various bioengineered systems to model and study the intestinal epithelium. Theses microfabricated *in vitro* models may constitute an alternative to current approaches for studying the fundamental mechanisms governing intestinal homeostasis and pathologies, as well as for *in vitro* drug screening and testing. Herein, we review the recent advances in bioengineered *in vitro* intestinal models.

Keywords

Intestine, 3D models, tissue engineering, microphysiological systems

Date received: 5 October 2020; accepted: 12 December 2020

Introduction

The intestinal epithelium is a multitasking tissue hosting multiple different cell types that ensure the digestion of aliments and protects the body from toxic microorganisms and carcinogens present in the luminal content. It is the fastest renewing epithelium in the body, with a complete renewal every 4–5 days.[1] The microenvironment of the intestinal epithelium is complex and dynamic. It is characterized by a specific 3D architecture, an ensemble of biochemical gradients and mechanical cues that together strongly affect cellular behavior.[2,3] Over the years, cell lines derived from tumors and, more recently, primary intestinal cells have been used extensively as *in vitro* models to study intestinal physiology and disease. However, most of these models do not faithfully recapitulate key *in vivo* features. In this context, there is a growing interest in combining tissue engineering and microfabrication techniques in an interdisciplinary approach to create more relevant tissue models. Compared to conventional 2D or 3D models, these so-called "microphysiological systems" provide more sophisticated and relevant systems allowing controlled and standardized production.[4,5] We will focus here on bioengineered systems developed to accurately recreate key features of the intestinal environment, such as the 3D architecture, mechanical stimulation or biochemical gradients.[6,7] These models have the potential to increase our understanding of human intestinal physiology and disease, and may represent an interesting alternative to animal models for drug screening. After an introduction on intestinal physiology and pathology, we provide an overview of the current approaches to study the intestinal epithelium *in vitro* and of the recent advances in the development of bioengineered model systems.

Function, cell biology and physiopathology of the intestine

The intestine is the longest organ of the digestive tract that extends from the stomach to the rectum. The main function of the intestine is digestion, namely the degradation of food released from the stomach, and the absorption of nutrients and water from the intestinal lumen into the

[1]LBCMCP, Centre de Biologie Intégrative, Université de Toulouse, CNRS, UPS, Toulouse Cedex, France
[2]LAAS-CNRS, Université de Toulouse, CNRS, Toulouse, France

*Equal contribution.

Corresponding author:
Arnaud Besson, LBCMCP UMR5088, Centre de Biologie Intégrative, Université de Toulouse, CNRS, UPS, 118 route de Narbonne, Toulouse Cedex 31062, France.
Email: arnaud.besson@univ-tlse3.fr

Creative Commons Non Commercial CC BY-NC: This article is distributed under the terms of the Creative Commons Attribution-NonCommercial 4.0 License (https://creativecommons.org/licenses/by-nc/4.0/) which permits non-commercial use, reproduction and distribution of the work without further permission provided the original work is attributed as specified on the SAGE and Open Access pages (https://us.sagepub.com/en-us/nam/open-access-at-sage).

blood circulation. The intestine is divided in two parts: the small intestine—itself subdivided into three segments, duodenum, jejunum and ileum—and the large intestine or colon.[8] While the small intestine is the main site of nutrient and metabolite absorption, the essential function of the colon is the recovery of water and electrolytes. The intestine is also an effective barrier against toxic or carcinogenic microorganisms present in the lumen of the digestive tract.[1,9] The intestinal epithelium is submitted to persistent aggression from the harsh luminal environment but also from mechanical abrasion and pH variations, leading to a high rate of cell deaths, with almost 10^{11} epithelial cells lost every day in the human intestine.[10] To compensate for this loss and ensure barrier integrity, the epithelium is continuously renewed with a turnover rate of 4 to 5 days, making the intestinal epithelium the tissue with the fastest renewal in the adult.[1,9] Additionally, both small intestine and colon play a crucial role in immunity by hosting the microbiota. The gastrointestinal tract is the primary site of interaction between microorganisms and the immune system.[11] The gut houses approximately 10^{14} microbes, mainly bacteria but also viruses, archaea and eukarya in the lumen and the mucosa. The exact composition of the microbiota strongly affects intestinal homeostasis and is unique to every individual, depending on the luminal environment (pH, nutrients, and probiotics). These microbes interact with the lymphoid tissue (Peyer's patches in the small intestine) and the host immune system.[11,12]

Histologically, the intestinal wall is composed of four layers: mucosa, submucosa, muscularis propria and serosa.[8] The serosa, or adventitia, is an external layer of loose connective tissue containing blood vessels and nerves. The muscularis consists in two layers of smooth muscles: the inner circular layer and the outer longitudinal layer. Contraction of this muscle wall creates the peristaltism that allows mixing and propulsion of the alimentary bolus through the intestine. This motion is coordinated by the enteric nervous system, comprising neurons and glial cells.[13] Inner from the muscularis, the submucosa is a connective tissue composed of stromal cells and of a dense network of arteries and lymphatic vessels required for nutrients absorption. Finally, the mucosa, lining the lumen, ensures the absorption function. It is composed of an epithelium supported by a connective tissue, the lamina propria, and the muscularis mucosae, a thin layer of smooth muscle, responsible of local movements and mucosa folding.[8]

The mucosa of each organ displays a specific organization necessary for their function. To maximize the absorption surface in the small intestine, the epithelium is organized in crypt and villus structures. Villi are projections of the epithelium into the lumen of the digestive tract. The base of each villi is surrounded by at least six crypts, called Lieberkühn crypts, which are invaginations of the epithelium into the lamina propria.[2] Villi are covered by a single layer of postmitotic differentiated epithelial cells, while proliferative intestinal stem cells (ISCs), named CBCs for crypt base columnar cells (identified by Lgr5), reside at the bottom of the crypts, where they are protected from aggression related to digestion. Inside the crypts, stem cells divide regularly to generate progenitors, the transit-amplifying cells that in turn divide four to five times, differentiating into mature epithelial cells while migrating to the base of villi.[1,2] In the villi, cells are fully differentiated and no longer divide. The upward migration continues and after 3 to 5 days, cells reach the top of the villi, where they shed into the gut lumen and die by anoïkis (apoptosis induced by loss of anchorage to extracellular matrix). In the colon, the mucosa lacks villi and is arranged in crypts with a flat luminal epithelial surface. The intestinal epithelium consists of at least six terminally differentiated cell types that all originate from the same stem cells. They can be divided in two lineages and each of them has a specialized function.[14] The absorptive lineage comprises enterocytes that represent up to 80% of intestinal epithelial cells and are responsible for nutrient uptake.[15] Enterocytes are highly polarized cells characterized by an apical brush border carrying microvilli that increase the exchange surface. The absorptive lineage also includes Microfold or "membranous" M cells, overlying the lymphoid tissues (Peyer's patches), which sample the intestinal lumen and transport antigens to the lymphoid cells underneath.[16] The secretory lineage comprises four cell types: Goblet cells are scattered throughout the epithelium and produce the protective mucus layer covering the intestinal surface.[17] Paneth cells are located at the bottom of the crypts and participate in the epithelial stem cell niche by secreting growth factors and presenting ligands to ISCs.[2] They also regulate the microbiota by secreting antimicrobial peptides.[2] Enteroendocrine cells, representing approximately 1% of epithelial cells, are chemosensory cells that regulate satiety, motility, immunity or inflammation by secreting a wide range of hormones in the bloodstream upon stimulation.[18] Finally, Tuft cells are rare cells ($<$0.4%) involved in antiparasitic type-2 immunity.[19]

Intestinal homeostasis and tissue integrity largely depend on ISC maintenance. ISCs are finely regulated by the complex microenvironment of the intestinal epithelium. This microenvironment results from the secretion of growth factors, cytokines and metabolites by surrounding mesenchymal and epithelial cells that constitute the niche.[3,13] In addition to these biochemical cues, physical signals such as extracellular matrix (ECM) composition, substrate stiffness, topography and mechanical stimulation also strongly influence stem cells. The microenvironment changes along the crypt-villus axis, with abundant proliferative signals in the crypt and differentiation signals increasing along villi in inverse gradients.[20] For example, growth factors that favor stemness, proliferation and self-renewal, such as Wnt,[21] EGF[22] and Notch,[23] are present in decreasing gradients from crypt bottom to the

top of villi, while BMP activity, which promotes differentiation, increases along villi.[24] ECM composition also changes along the crypt-villus axis. The ECM of the intestinal epithelium is mainly composed of fibronectin, laminin and collagen IV,[3] and while collagen IV is uniformly distributed, different laminin subtypes are regionally expressed, with laminin α2 enriched in villi and laminin α5 and α3 in crypts.[25] Fibronectin also displays a specific distribution and is preferentially expressed in crypts.[26] These changes in matrix composition also suggest variations of stiffness, however due to technical limitations it has not been directly measured *in vivo*. Measurements on *ex vivo* samples showed that healthy intestinal ECM has a Young modulus around 2.9 kPa, whereas tissues obtained from Crohn's disease patients exhibited an increased rigidity, with a Young modulus of 16 kPa, suggesting significant ECM remodeling due to inflammation.[27] Interestingly, increased fibronectin deposition has been described in chronic inflammatory disease patients.[28] In addition to stiffness, other mechanical cues can affect cellular behavior, notably muscular contractions that generate peristaltism (frequency of 3/s in postprandial with an amplitude of 20–24 mmHg) and shear stress due to flow rate (0.7–3.0 mL/min).[29]

The balance and competition between chemical or physical signals allows the coordination of intestinal epithelial cell proliferation, localization, migration and differentiation in order to maintain tissue homeostasis[3] (Figure 1). Disruption of this equilibrium can lead to an alteration of the epithelium and the development of several pathologies, including inflammatory bowel disease (IBD) or colorectal cancer. IBD is a heterogeneous set of inflammatory disorders of the gastrointestinal tract, including two main clinical pathologies: Crohn's disease and ulcerative colitis.[30] Although, the etiology and pathogenesis of IBD is poorly understood, it is accepted that IBD results from an uncontrolled immune response against environmental factors in genetically susceptible individuals.[31,32] IBD represents a major public health problem and an important financial burden, with over 6.8 million people affected worldwide.[32,33] Current therapeutic strategies are based on the use of anti-inflammatory or immunosuppressant molecules.[34] However, despite a high rate of clinical remission, current drug treatment still fail in a significant number of patients and 33% of Crohn's Disease patients and 11% of ulcerative colitis patients require surgical resection within 5 years of diagnosis.[35,36] Moreover, as a consequence of chronic intestinal inflammation, patients with IBD are two to sixfold more likely to develop colorectal cancer than the general population.[37,38] Colorectal cancer is the third most common type of cancer and the fourth cancer-related cause of death worldwide, with over 1.2 million new cases and 900,000 deaths per year, with a growing incidence in developing countries.[39] The cell of origin for most colorectal cancers is thought to be a stem cell. Transformation of this cancer stem cell results from the accumulation of genetic mutations (primarily APC mutation followed by mutations in K-Ras, p53, PTEN and/or SMAD4) leading to hyperproliferation and deregulation of the stem cell compartment.[40] Conventional therapeutic strategies frequently fail due to tumor heterogeneity and resistance of cancer stem cells to treatments, causing relapse of the disease.[41]

Thus, there is a pressing need to improve our understanding of the mechanisms underlying theses pathologies and to identify new targets and therapeutic strategies. Furthermore, despite very promising results in pre-clinical studies in animals models, over 90% of clinical trials fail,[42] suggesting that these models do not faithfully recapitulate human physiology and disease. In addition, the use of animals in experimentation raises ethical issues, and current guidelines and legislation recommend to follow the "3R" principles: "Replace, Reduce, and Refine".[43] For all these reasons, *in vitro* models are already widely used for fundamental studies or drug testing and toxicology, as they provide a simpler context with better controlled and repeatable conditions to investigate cellular responses.[44] Nevertheless, the limitations of current *in vitro* models based on cancer cell lines has motivated the use of adult primary cells or induced pluripotent stem cells (iPSCs) carrying specific mutations that better recapitulate human pathologies, notably with the development of organoids. However, these *in vitro* models also present some disadvantages (see below). Therefore, there is a real need to create better *in vitro* systems.

Current approaches to study the intestinal epithelium *in vitro*

The epithelial cells most frequently used for *in vitro* studies are cell lines derived from colorectal cancers. These cell lines are highly proliferative and relatively easy and cheap to use, making them very useful for mechanistic studies, toxicology assays or high throughput screening approaches. A large number of colorectal cancer cell lines are now available, such as HT-29, Caco-2, T84, SW480 and many others, but most of them exhibit high variations in term of differentiation state, metabolism and proliferation, possibly due to the diversity of tumors from which they originate and the different set of mutations they carry.[45] Among them, Caco-2 cells represent the most widely used model for drug permeability and absorption experiments, as well as for differentiation. Caco-2 cells spontaneously differentiate in a polarized epithelium upon reaching confluence, starting around 7 days after seeding and completing at 21 days of culture. These cells then exhibit characteristics similar to enterocytes, such as apical brush borders, tight junctions and expression of several enterocyte-specific enzymes and transporters.[46,47] However, Caco-2 cell monolayers do not reproduce the diversity of cell types present in the primary tissue. To

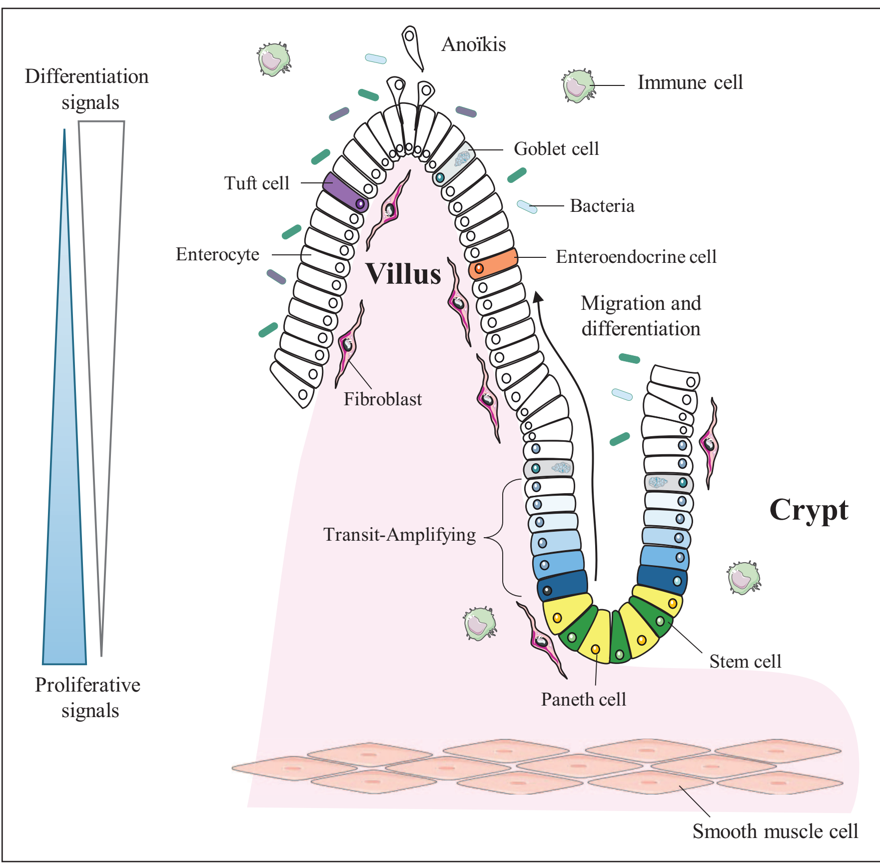

Figure 1. The intestinal epithelium. The intestinal epithelium is organized in crypts and villi. Proliferative stem cells are located at the bottom of the crypts, where they divide and give rise to progenitors or transit-amplifying cells, which rapidly divide and differentiate into mature epithelial cells. During differentiation, cells migrate upward towards the top of villi, where they are extruded in the gut lumen and die by anoïkis. Homeostasis is supported by the microenvironment, which changes along the crypt-villus axis, with proliferative signals at the bottom of crypts and differentiation signals increasing in villi. The microenvironment results from all the components supplied by epithelial cells but also intestinal mucosa and lumen. The components of the mucosa include the extracellular matrix and the underlying stromal cells. Conversely, the lumen of the digestive tract is populated with the microbiota, which can also have a strong impact on the epithelium, and is exposed to many pathogens.

address this, they have been co-cultured with the mucus producing HT29-MTX cell line, thus mimicking both enterocytes and goblet cells.[48,49] Nevertheless, these established intestinal cell lines are not always relevant since they are mostly similar to enterocytes, and therefore are not representative of the heterogeneity of the entire intestinal epithelium. Another drawback of these cell lines is their difference to normal epithelial tissue. Indeed, due to their cancerous origin they harbor multiple gene mutations, for instance, Caco-2 cells are aneuploid and carry a mutated p53 gene.[50]

A major breakthrough occurred in 2009 with the development of the intestinal organoid model and the identification of culture conditions and growth factors required to sustain ISC proliferation and differentiation.[40] Organoids are defined as an *in vitro* 3D organotypic culture obtained from primary tissue, embryonic stem cells or induced pluripotent stem cells, capable of self-renewal and self-organization, and exhibiting similar organ functionality as the tissue of origin.[4,51] Intestinal organoids may be obtained from isolated $Lgr5^+$ ISCs or dissociated crypts embedded in Matrigel (an ECM rich in laminin and collagen) and overlaid with a minimum culture medium. This medium is supplemented with key niche signals, R-Spondin (a Wnt agonist that maintains stem cell population), Noggin (a BMP inhibitor that limits differentiation) and EGF (epidermal growth factor, to promote cell proliferation) that allow reproducing the proliferation and differentiation program that generates the intestinal epithelium. Once in Matrigel, stem cells proliferate, differentiate and self-organize, forming 3D spherical structures with crypt-like domains containing stem cells and Paneth cells fueling villi-like regions containing all mature cell types found in the intestinal epithelium. These crypt/villi-like domains form a central lumen containing dead cells extruded from the constantly renewed epithelial layer.[40,52] Organoids represent a relevant system recapitulating the heterogeneity and renewal of the

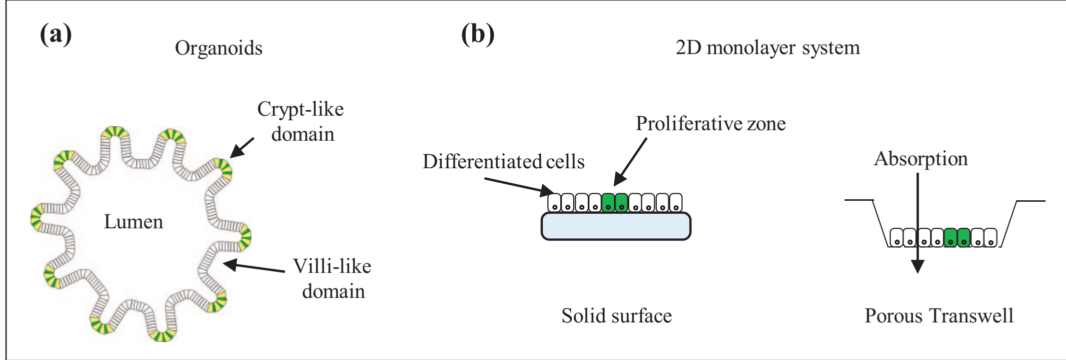

Figure 2. Current *in vitro* models to study the intestinal epithelium. Several *in vitro* models have been developed to study the intestinal epithelium. (a) Organoids are 3D organotypic cultures obtained from dissociated intestinal crypts, in which cells self-organize, with an enrichment of stem cells in crypt-like domains and of differentiated cells in villi-like regions along the central lumen. (b) 2D self-renewing monolayers have been obtained from 3D organoids, allowing easy access to both basal and luminal compartments.

intestinal epithelium, with great potential for a wide range of applications, from basic research to translational applications, such as disease modeling, drug testing or host-microbe interactions[53,54] (Figure 2(a)). Organoids can also be generated from iPSCs, and then called iHIO (induced Human Intestinal Organoids).[55] For this, human pluripotent stem cells are first differentiated into definitive endoderm in 2D by Activin-A and then intestinal lineage specification is induced with Wnt3a and FGF-4 treatment, leading to the formation of primitive gut tubes.[55] Finally, gut tubes are transposed in Matrigel and cultivated in minimum culture medium. In comparison to organoids obtained from adult stem cells, iHIOs develop crypts and villus-like structure and present the advantages of possessing a mesenchyme surrounding the epithelial layer.[56] Furthermore, during their generation, iHIOs recapitulate the developmental program of the intestine, making this system a model of choice to study human gastrointestinal development.[56] However, iHIOs exhibit immature features, with a more fetal phenotype and require further maturation *in vitro*[57] or by transplantation *in vivo*.[58] Overall, we dispose of various cellular models to study the gastrointestinal tract, and the choice of the source of cells will depends on the question and application (Table 1).

Despite many advantages, the organoid model has limitations, including the lack of native environment and the inability to reproduce biochemical gradients and biophysical cues (matrix stiffness), and the difficulty to apply mechanical stimulation such as peristaltism or shear stress in the Matrigel matrix. Furthermore, organoids in the same culture are very heterogeneous in terms of size, shape and viability, making phenotypic studies difficult.[4,5] In addition, their spherical architecture is a major limitation since the apical lumen is inaccessible, and studies based on host-pathogen interaction require using microinjection, which is technically challenging.[59] To overcome this issue, 2D self-renewing monolayers derived from 3D organoids have been developed (Figure 2(b)). The main difficulty is the selection of a suitable substrate promoting cell adhesion and stem cell maintenance, while preventing organoid formation. Various system have been used such as thin layers of gelatin,[60] thick layers of collagen I hydrogel (>1 mm)[61] or thin coating of Matrigel on solid surface[62] or porous Transwell scaffold.[63] The monolayers exhibit a polarized morphology and express markers of all differentiated cell types.[61–63] Interestingly, similar to 3D organoids, cells in monolayer self-organize in stem/proliferative zones and differentiated cells areas.[61–63] When cultivated on porous insert supports, these systems provide access to both luminal and basal sides allowing the monitoring of absorption of drugs, nutrients or microorganisms, as well as the effect of host-pathogen interactions on epithelium integrity or the incorporation of other cell types such as immune cells. Thus, epithelial monolayers represent a simple and relevant model to study these complex interactions. However, these systems lack the 3D architecture, biochemical gradients or dynamic mechanical forces of the native microenvironment in the primary tissue.

Development of bioengineered systems

Due to the limitations of current *in vitro* models to study the intestine, there is growing interest for tissue engineering and the use of microfabrication techniques to develop more relevant culture systems in a more controlled and standardized manner. Microphysiological systems (MPSs) are defined as *in vitro* models recapitulating key features of *in vivo* organ function by using specialized microenvironment systems, such as 3D matrices, multicellular architecture and/or microperfusion.[64,65] In the case of the intestine, key characteristics of the native environment that should be reproduced include the specific ECM composition, 3D architecture, gradients present along the crypt-villus axis, and mechanical stimulation of the tissue (shear stress and peristaltic contractions).

Table 1. Sources of cells to study intestinal physiology.

Cell source	Culture model	Advantages	Disadvantages	Applications
Adult stem cells Dissociated fresh crypt Dissociated organoids	Organoids 3D scaffolds Gut-on-chip 2D monolayer	Human or murine origin Heterogeneity	Adult stem cells Only epithelial cells	Genetic disease Drug screening Host/pathogen interaction Adult epithelial function Adult intestinal stem cell biology Tissue engineering
Pluripotent stem cells (iPS)	Organoids Gut-on-chip	Human origin Presence of mesenchyme Recapitulate the development	Immature features require maturation in vivo or *in vitro*	Genetic disease Drug screening Host/pathogen interaction Organ development Tissue interaction Tissue engineering
Cancer cell lines Caco-2 Caco-2/HT29 SW480	2D monolayer transwell 3D scaffolds Gut-on-chip	Human origin Easy and more affordable	Lack of heterogeneity Cancer origine	Cellular and molecular biology Absorption and toxicology assays Host/pathogen interaction Tissue engineering

Selecting a material to reproduce ECM

The role of the biomaterial is to provide the biochemical, topological and mechanical cues allowing adhesion and supporting proliferation and differentiation of epithelial cells. So far, ISC cultures have mostly relied on materials of natural origin such as Matrigel[40] or collagen I.[61,66,67] Despite many advantages and notably their ability to support the growth of many cell types, natural matrices are heterogeneous and their variability from batch to batch can significantly affect experimental reproducibility. Additionally, the gelation kinetics and stiffness of these matrices are difficult to control.[68] An interesting alternative is the use of synthetic materials as ECM replacements, and the most widely used are synthetic hydrogels, particularly those based on covalently cross-linked polymers, such as polyethylene glycol (PEG).[69] Synthetic hydrogels offer well-defined structures and compositions that allow reproducible elaboration of cell culture models.[68,70] They also provide more flexibility in terms of chemical and mechanical properties as the composition of the material (molecular weight, stoichiometry. . .) can be easily tuned.[70] However, these polymers are often inert, offer poor adhesive properties and are non-degradable, which severely limits cellular colonization and their use in regenerative medicine. Polymers can be modified to generate semi-synthetic or biohybrid hydrogels that aim to combine the advantages of both natural and synthetic hydrogels.[68,69] Semi-synthetic materials are mostly made of adhesion peptides (e.g. RGD peptides) or ECM molecules (fibronectin, laminin, collagen) grafted on a synthetic polymer backbone.[71,72] They also can be produced by co-polymerization between the polymer and the biological conjugate.[73] In addition, these materials can be modified to integrate matrix metalloproteinase (MMP) target sites to make them susceptible to cell-mediated degradation with tunable sensitivity.[69,72] In addition, growth factors such as VEGF[72] or EGF[74] can be incorporated or cross-linked in hydrogels to promote cell colonization, proliferation, migration or differentiation. The flexibility of these biomaterials is a major advantage to decompose the complexity of the ECM and to uncouple mechanical cues from biochemical aspects to understand cellular behavior.

Several recent studies devoted to the intestinal epithelium have focused on the use of biosynthetic materials to generate organoids and replace Matrigel to improve reproducibility and control over physical properties.[69] Studies reported the generation of organoids from adult ISCs embedded in customized PEG-based hydrogel.[75,76] By varying mechanical properties and adhesion molecule composition, it was demonstrated that high matrix stiffness (1.3 kPa) promotes ISC expansion through activation of the YAP/Hippo signaling pathway, while soft and degradable matrices and the presence of laminin $\alpha1\beta1\gamma1$ were needed for cell differentiation and organoid formation.[75,76] Similarly, human intestinal organoids have been generated from iPSCs or human embryonic stem cells embedded in PEG-4-MAL (four arm PEG-maleimide) hydrogels.[77,78] Importantly, the presence of MMP-degradable sites and RGD adhesion ligands were required for organoid survival. The proportions of the different components were tuned to obtain optimal conditions for ISC expansion similar to those obtained in Matrigel. For instance, a 2 mM concentration of RGD peptides, a Young modulus of 100 Pa and the presence of degradable sites were identified as key conditions required for intestinal organoid culture.[77,78] The modular structure of the hydrogel was established to obtain an injectable delivery system that could transfer encapsulated organoids to mucosal wounds in mouse intestine, facilitating engraftment onto injured tissues.[77,78]

These pioneering studies have demonstrated the benefits of synthetic materials to easily modulate ECM for organoid generation and shown their potential for generating more controlled organoid cultures. Furthermore, the synthetic composition of these hydrogels facilitates *in vivo*

delivery of intestinal organoids or ISCs, thus showing potential for regenerative therapy in intestinal disease. However, these 3D cultures still lack biochemical gradients, dynamic forces and a defined 3D architecture. To overcome this gap, multiple bioengineered systems have been developed recently to study the intestinal epithelium using various biofabrication techniques.

Technical approaches to reproduce 3D architectures

Tissue engineering and MPS often rely on the reproduction of tissue architecture to allow guided cell organization. Indeed, it appears that the 3D architecture strongly influences cell proliferation, differentiation and tissue homeostasis.[79] Microfabrication describes the process of fabricating structures down to the micrometer scale. Initially developed for integrated circuit fabrication, it is now used in many applications, including the fabrication of MPSs. Microfabrication techniques commonly used for MPSs include photolithography, soft-lithography, 3D printing or microfluidics[80] (Figure 3). Photolithography and soft-lithography (by molding) have been used in several studies to create 3D intestinal scaffolds, proving the advantages and utility of microfabrication techniques in the development of *in vitro* culture models.[81,82]

Photolithography is a micropatterning technique used to transfer a pattern to a photosensitive resist by exposure to UV light through a photomask.[83] Briefly, a photosensitive polymer (photoresist) is spin coated to form a uniform thin film on a substrate (usually a silicon wafer), and aligned with a photomask that consists of opaque features (typically chrome) on a transparent substrate. The ensemble is then exposed to UV light through the photomask which protects opaque regions while exposing other areas according to the design of the photomask. In the case of negative photoresists, polymerization occurs in exposed areas. Non-polymerized regions become soluble in a developer solution and dissolve away during the development step, thus leaving the desired pattern made of resist on the substrate (Figure 3(a) and (b)). Photolithography is a powerful technique to create patterns with a submicron resolution. This technique has been widely used to create micropatterned substrates to study the impact of topology on cell proliferation and fate. However, creating complex 3D structures with high aspect ratios and curvatures on hydrogels using this approach remains challenging.[83,84]

As an alternative to photolithography, soft-lithography is a set of techniques relying on the fabrication of a soft elastomeric "master," generally a PDMS (PolyDimethylSiloxane) stamp, typically fabricated by replica molding on a patterned substrate obtained by photolithography. In this case, a prepolymer solution is deposited on the photoresist mold, cured, and then separated by peeling them apart. This method allows replicating the structures initially present on the substrate in a PDMS stamp. This stamp can be used to transfer 2D patterns of biomolecules onto a surface (microcontact printing). Similarly, microtransfer molding combines the advantages of microcontact printing and replica molding to produce simultaneously functionalized microstructures on the surface of the substrate.[84,85] Soft lithography techniques are widely used, inexpensive, and allow fast production (Figure 3(c) and (d)). However, due to the replication by molding, architectures are mainly restricted to 2D or 2.5D geometry, and the numerous fabrication steps needed to obtain the PDMS master are limiting the expansion of this technology.

A promising alternative to generate 3D scaffolds is 3D printing. 3D printing is an additive manufacturing technique in which a three-dimensional object is built by successively adding thin layers of material. The object is designed digitally with a computer aided-design (CAD) program, and then converted to an STL (Standard Tesselation Language) file. This file is then processed by the 3D printer software, which divides the object in a sequence of thin 2D horizontal slices (5–100 µm thick depending on the 3D printing technique), which are then successively printed to create the object[86] (Figure 3(e) and (f)). Several 3D printing techniques are available and used for bioengineering, including stereolithography, laser assisted printing, inkjet printing or microextrusion printing.[87] The progressive adaptation of 3D printing toward biomaterials has promoted the development of bioprinting technologies that allow the fabrication of 3D architectures from bioinks incorporating living cells. Despite their price and some limitations in the material libraries,[88] these methods offer the opportunity to build tissue constructs by controlling the spatial distribution of matrices, biomolecules or cells. In that case, living cells embedded into a biomaterial (then called bioink) is printed in 3D. Once printed, cells proliferate, spread and migrate, and eventually remodel the printed structures. This mechanism has been introduced in the concept of 4D printing where the fourth dimension represents the evolution of the biological object over time.[87,89]

It has been shown that 3D printing is well adapted for the creation of 3D scaffolds for cell cultures with high reproducibility, but only a few studies so far have reported the use of 3D printing to create intestinal models. Even though the resolution offered by these technologies permits the fabrication of intestinal architecture, these techniques are often not compatible with the printing of living cells. Conversely, in terms of resolution, current bioprinting techniques do not offer the possibility to create scaffolds with dimensions and geometry mimicking intestinal topography. Nevertheless, bioprinting can be very useful to recreate the mesenchyme and study cell-cell interactions. The approach and technique used depend therefore on the desired application.

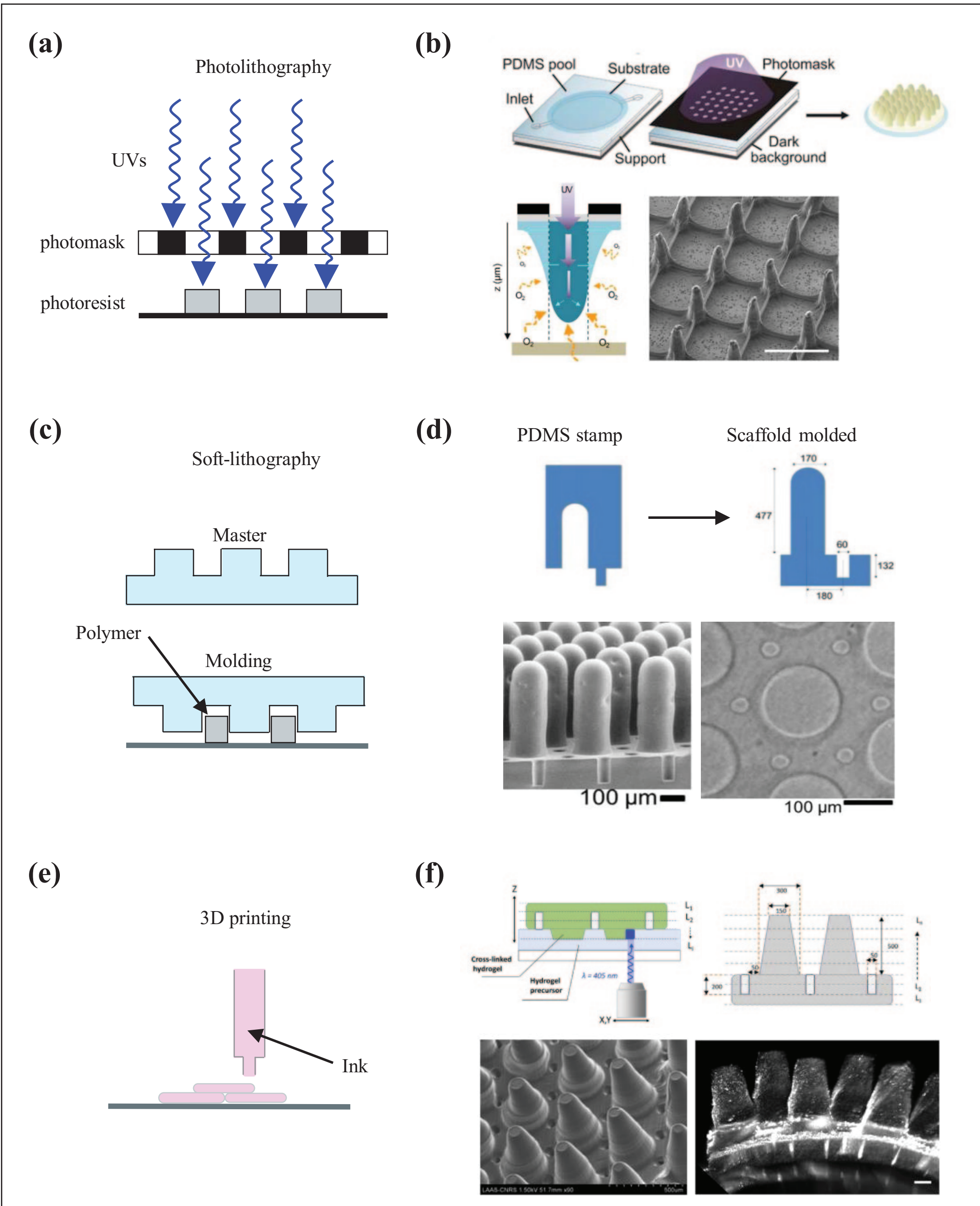

Figure 3. Microfabrication techniques in bioengineered intestinal models. (a, b) Photolithography is a microfabrication process using UV light to transfer geometric patterns from a photomask to a photosensitive resist on a substrate. Photolithography has been used to generate scaffolds reproducing intestinal villi in PEG hydrogel (adapted from Castano et al.[82]). (c, d) Soft-lithography is a technique using an elastomeric stamp or master to fabricate or replicate 2D or 3D patterns. Soft-lithography has been used to replicate the intestinal architecture in a collagen scaffold molded using a PDMS stamp (adapted from Wang et al.[81]). (e, f) 3D printing is an additive manufacturing technique that can be used to create complex 3D structures containing or not living cells. Using a stereolithography printing technique, a 3D scaffold mimicking the topography of the intestinal epithelium was made in a PEG-DA/acrylic acid/ECM mix (adapted from Creff et al.[101]).

Reproduction of intestinal epithelium topography

As described earlier, the intestinal epithelium is a highly polarized tissue displaying a particular 3D architecture, with a crypt-villus organization. To study the impact of this particular architecture on cellular behavior, various microfabrication processes and materials have been used to generate 3D scaffolds mimicking this topography.

One of the first study reported the generation of scaffolds reproducing intestinal villi in collagen I hydrogel using a combination of molding techniques.[90] Caco-2

cells were seeded and grown for up to 3 weeks on these scaffolds. Morphological similarities were observed between cells grown in 3D and human villi.[90] These scaffolds were subsequently integrated in an insert system, and it was shown that this system improves the correlation between the Caco-2 cell model and human native small intestine in drug permeability experiments, with trans-epithelial electrical resistance (TEER) values closer to those physiologically observed in the tissue (Figure 4(b)).[91] Additionally, growing Caco-2 cells on 3D collagen scaffolds promotes the expression of differentiation markers and the expression of mucins such as MUC17 was induced several fold compared to monolayer culture.[92] This system has been used to assess drug permeability[91] and to study the role of MUC17 in antibacterial response,[92] showing the relevance of these 3D models for studying intestinal physiology. However, the cells progressively degrade and invade the collagen I structure and after 3 weeks, the observed dimensions of the villi were reduced to almost half of the original size and multiple cell layers were observed.[90,91]

To avoid this limitation, scaffolds in synthetic materials have been developed. Using the same molding techniques, PLGA (poly-lactic-glycolic acid) villi scaffolds have been created and integrated in porous insert.[93] This platform supports the growth of Caco-2 cells co-cultured with mucus-producing HT29-MTX cells. As previously described, the 3D architecture promotes the expression of differentiation markers and this effect was enhanced in presence of an EGF gradient along the vertical axis.[93] This system was adapted for the culture of primary intestinal cells obtained from murine dissociated crypts and the 3D topography promoted differentiation and spatial organization of intestinal cells, with more differentiation at the tip of villi than at their base.[93] This platform was used to study host/microbe interactions by co-culturing commensal or pathogenic bacteria with Caco-2 cells to evaluate the effects of probiotics, revealing that the 3D architecture induced preferential localization and adhesion of specific types of bacteria at the tip or near the base of the scaffolds, confirming the relevance of 3D model to mimic host/pathogen interaction.[94] However, this fabrication method is relatively complex and requires multiple steps, limiting routine use of this approach.

Recently, 3D microstructures mimicking intestinal villi were produced in a single fabrication step and moldless approach using a photolithography process (Figure 3(b)).[82,95] Scaffolds made of PEG-DA co-polymerized with acrylic acid and functionalized with extracellular matrices proteins were generated and integrated into inserts. It was demonstrated that these devices support adhesion and growth of Caco-2 cells and that 3D architecture greatly influences cells shape, polarization and differentiation.[82,95] This system provides an easy and rapid method to obtain 3D scaffolds with high aspect ratio and curvature. However, thus far this technology is not adapted to the creation of crypts.

Taken together, models recapitulating some features of intestinal topography have highlighted the pro-differentiative impact of villi-like architecture on cells. However, these scaffolds reproduced villi in absence of crypts, and therefore do not fully reproduce the *in vivo* topography. Several studies indicate that the presence of crypt structures strongly influences the differentiation of intestinal cells. The generation of crypt-like microwells on PDMS[96] or on collagen membranes[97] using photolithography has shown that Caco-2 cells grown on these substrates exhibit reduced expression of differentiation markers, increased metabolic activity[96] and lower TEER[97] compared to classic 2D cultures, suggesting that the crypt topography favors a stem cell-like phenotype.

In this context, 3D printing offers the possibility to produce complex microscale 3D structures with an architecture matching that of the intestinal epithelium. With recent progress in bioprinting, it is now possible to print living cells and a recent study has reported the development of a 3D model of intestinal tissue generated by bioprinting. This 3D tissue consists of two printed layers: a supporting layer of human intestinal fibroblasts and an epithelial layer containing human intestinal epithelial cells.[98] This model recapitulates some key features of the native tissue, such as cell polarization, tight junctions, expression of differentiation markers (villin, lysozyme or chromogranin A), and increased CYP450 activity, representing a promising model for toxicology studies in drug development.[98] However, in this model, the 3D architecture was not integrated and other studies have explored the possibility of recreating villi structures by bioprinting.[99,100] Using collagen-based bioinks, the 3D geometry was reproduced by printing a mesh structure for the crypt compartment and vertical protrusions to mimic villi. These models have been developed either with one bioink containing Caco-2 cells for the epithelium[100] or with two bioinks to generate an external Caco-2 cells layer that overlays the core of villi containing Human Umbilical Vein Endothelial cells (HUVECs) to reproduce capillary structures.[99] In both studies, either with one or two cell types, the cultures were more homogeneous and exhibited higher proliferation rate and expression of differentiation markers.

Scaffolds reproducing both crypts and villi have been generated using a photopolymerizable PEG-DA based hydrogel combined with high-resolution stereolithography 3D printing (5 µm resolution) (Figure 3(f)).[101] These scaffolds were seeded with Caco-2 cells, and it appears that both the hydrogel material and the 3D topology strongly influence cell behavior, as cells grown on hydrogel in 3D exhibited increased polarization and expression of enterocytes differentiation markers compared to 2D cultures.[101] This study highlighted the feasibility and benefits of 3D printing to fabricate complex 3D scaffolds.

A recent study described the generation of micropatterned collagen I scaffolds reproducing the crypt-villus architecture

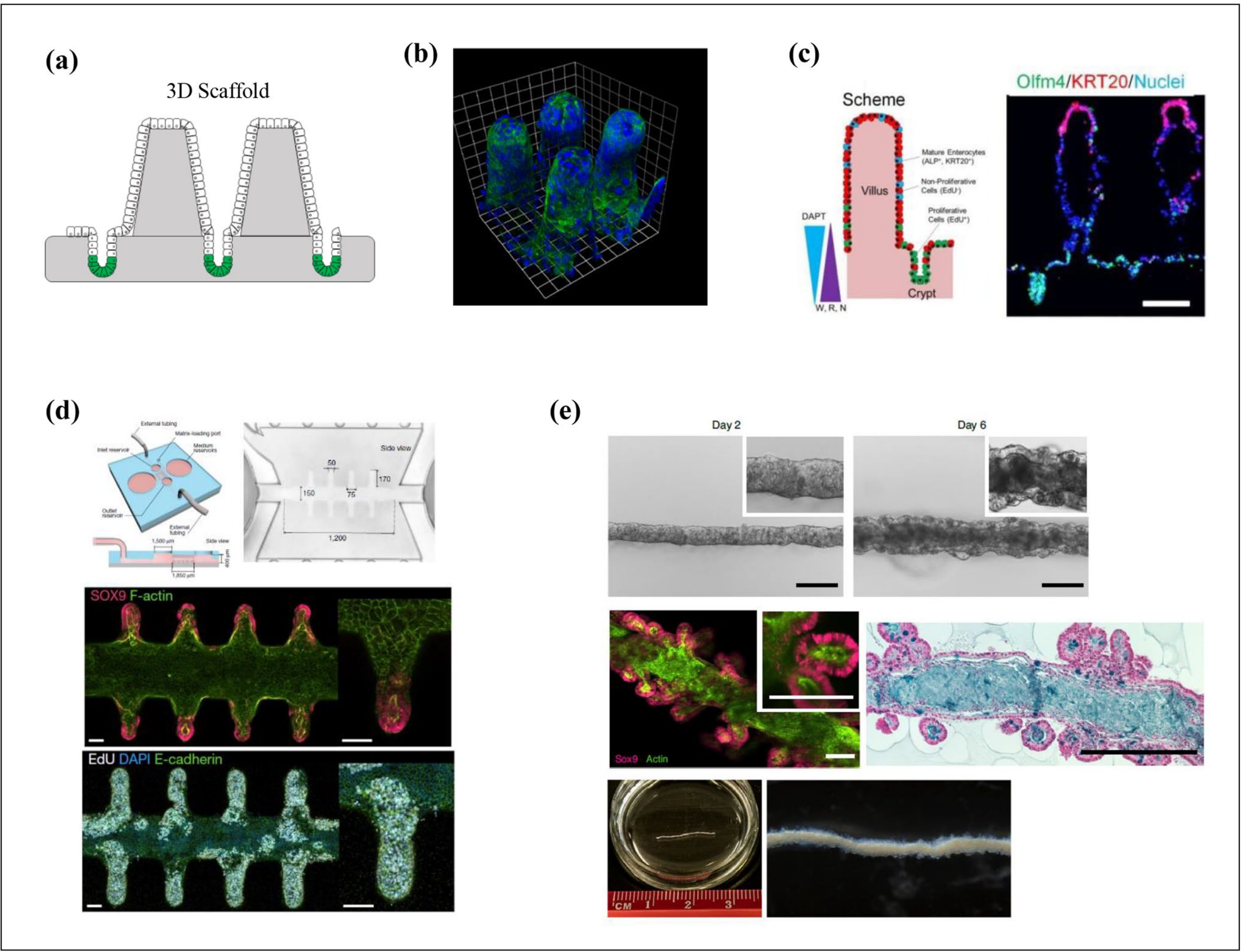

Figure 4. Reproduction of intestinal epithelium topography. Studying the impact of 3D architecture on cellular behavior requires the generation of 3D scaffolds reproducing tissue topography and allowing guided cell organization. (a) Schematic of 3D scaffolds used in bioengineered systems. (b) Fluorescence images (3D reconstruction and XY section) of Caco-2 cells grown on a 3D collagen I scaffold (adapted from Yu et al.[91]). (c) Schematic of the model reproducing the inverse growth factor gradients (N: Noggin; R: R-Spondin 3; W: Wnt3a) and fluorescence images of cross-sections showing intestinal epithelium with immature cells in crypts (Olfm4) and differentiated cells in villi (Krt20) (adapted from Wang et al.[81]). (d) Development of mini-intestine on chip, upper panel: schematic of microdevice developed for culture. Middle and bottom panel: Fluorescence images showing progenitors (Sox9) and proliferative cells (EdU) generating differentiated cells (adapted from Nikolaev et al.[102]). (e) Macroscale intestinal tissue obtained by bioprinting of intestinal organoids. Upper panel: Bright-field images of intestinal tube with formation of lumen and budding structure 6 days after printing. Middle panel: Fluorescence images of intestinal tubes showing progenitors cells (Sox9) and histological staining of alcian blue and Nuclear Fast Red showing mucus production and Goblet cells. Bottom panel: macroscopic images and intestinal tubes (adapted from Brassard et al.[103]).

by molding (Figure 4(c)). Collagen I was covalently cross-linked to prevent its degradation by cells, and the system was integrated in porous insert to recreate the opposite growth factor gradients, with proliferative signals in the basal reservoir (Wnt, R-Spondin, Noggin) and differentiation signals (DAPT, a Notch inhibitor) in the upper compartment.[81] This system allowed the growth and differentiation of primary human intestinal cells and revealed that in addition to 3D topography promoting cell organization, the presence of adequate chemical gradients was necessary for the segregation of cells in a stem/proliferative zone and to support unidirectional migration and differentiation along the crypt-villus axis.[81] This study highlighted the importance of recapitulating both architecture and biochemical environment. Altogether, these findings emphasize the idea that 3D topography directly contributes to cell differentiation and tissue function, and therefore that reproducing these features *in vitro* allows generating models more representative of the native tissue.

An interesting approach recently combined 3D micropatterning with the self-organization properties of cells.[102] This “mini-intestine” consists in a tubular scaffold in collagen I/Matrigel mix with an accessible lumen surrounded by microcavities that mimic the geometry of crypts. (Figure 4(d)). The structures were generated by laser ablation. The hydrogel scaffold was integrated in a microsystem that consists of a central chamber, for hydrogel loading and organoids culture,

flanked by reservoirs for cell loading and luminal perfusion. Dissociated intestinal organoids were seeded in the microchip and cells self-organized, with proliferative and immature cells exclusively in crypt-like regions while their progeny migrate and replenish the differentiated cells, like in the native tissue. Perfusion allows the continuous removal of dead cells, making possible to maintain these cultures without passage for several weeks, and provides easy access to luminal content for modeling pathogen interaction.[102] Even if this system only partially recapitulates the 3D architecture of the tissue and does not integrate mechanical stimulation, the use of guided cell organization allows the formation of a functional organoid-chip and thus shows a great improvement compared to classical organoids cultures, notably allowing access to the lumen, which is one of the main limitation.

Finally, the same group achieved a new technological breakthrough by producing macroscopic intestinal tubes using bioprinting.[103] This new bioprinting technology, named bioprinting-assisted tissue emergence (BATE) uses stem cells or organoids as building blocks. Thanks to a syringe-based extrusion method, the cells and organoids can be spatially arranged directly into an extracellular matrix composed of collagen I mixed with Matrigel (Figure 4(e)).[103] Dissociated organoids are printed at high density in a cylinder shape, they first condense into a thick tubular, and within a few days, they expand and fuse into a polarized and lumenized intestinal tube. After 4 to 6 days, the epithelial cells start to bud and form crypt-like structures fueling the differentiated domains of the tissue. These intestinal tubes respond to chemical stimulation (swelling induced by Forskolin or release of Paneth cells' granules in response to carbamylcholine), indicating that the tissue obtained recapitulates some functionalities of the organ *in vivo*. One of the major advantages of the BATE technology is the possibility to print multiple cell types. Stromal cells were printed next to epithelial cells, leading to an increased diameter of the lumen, allowing perfusing of the intestinal tube.[103] However, this system relies on cellular self-organization and even if the macroscopic arrangement is reproduced, the microscale topography with high aspect ratio crypt/villi architecture is not reproduced in this model.[103] This model also uses natural ECM, which precludes its use in regenerative medicine.

Implementing mechanical stimulation in organ on chip systems

The term organ-on-chip was introduced by Donald Ingberg in 2010 and defined as "microfluidics devices for culturing cells in continuously perfused, micrometer sized chambers in order to model physiological function of tissues and organs".[104] Microfluidics refers to "the science and technology of systems that process or manipulate small amounts of fluid (10^{-8}–10^{-9} L), using channels with dimensions of tens to hundreds of micrometers".[105] Due to the geometrical confinement and the predominance of viscosity, fluid flow is laminar and no mixing occurs between adjacent streams, allowing the generation of gradients solely by passive diffusion.[83] The simplest system consists of a microfluidic chamber seeded with one cell line perfused uniformly. This type of system has been used to study various mechanisms, such as the impact of biochemical gradients,[106] stem cell differentiation[107] or axon guidance.[108] Since then, more sophisticated systems have been developed, composed of several channels interconnected with porous membranes, and including several cell types to mimic the interface between tissues.[83]

Organ-on-chips have been used to reproduce the dynamic mechanical stimulation created by shear stress of the luminal content and peristaltism from the muscle wall, which are key features of the intestinal microenvironment. The "gut-on-chip" was developed initially with a cancer cell line in 2012 and is made of a central chamber subdivided into two channels by a 30 μm ECM-coated porous PDMS membrane and surrounded by two lateral vacuum chambers, resulting in cyclic deformation of the flexible membrane (10% stretch, 0.15 Hz) to mimic peristaltic motions (Figure 5(a)).[109] Caco-2 cells were seeded on the PDMS membrane and the chamber was perfused at a flow rate of 30 μL/h, mimicking shear stress. Under these dynamic conditions, Caco-2 cells spontaneously underwent villus morphogenesis, adopting a specific organization with proliferative cells localized near the membrane and cells expressing differentiation markers, such as villin or mucin, localized in the villi-liked structures (Figure 5(b)).[109,110] This system allows decoupling the effect of shear stress from that generated by cyclic stretching forces, and studying their influence separately or collectively on the epithelium. Interestingly, it was shown that fluid flow plays a critical role in the initiation of villi morphogenesis.[109,110] Thanks to continuous fluid flow, it is possible to co-culture commensal microbes and pathogenic agents (the VSL#3 clinical probiotic formulation containing eight microbial strains) in direct contact with epithelial cells. Transcriptomic analyses revealed that Caco-2 cells in gut-on-chip co-culture with normal microbes exhibit a gene expression profile that is more similar to normal ileum compared to static Transwell cultures.[111] This system was then used to mimic complex microbiome-immune interaction in chronic inflammatory diseases such as IBD (Figure 5(c)). Isolated human peripheral blood mononuclear cells (PBMCs) were introduced in the lower channel to mimic the immune compartment. Addition of endotoxin to the luminal compartment induced the secretion of pro-inflammatory cytokines in the lower channel leading to injury of the epithelial tissue, thus mimicking inflammation-induced damage.[111] More recently, the gut-on-chip system was adapted for the culture of human intestinal organoids obtained from iPSCs[112] and for co-culture of human primary intestinal epithelial cells in the upper compartment with human primary intestinal vascular endothelial cells to recreate both an epithelial luminal compartment as well as

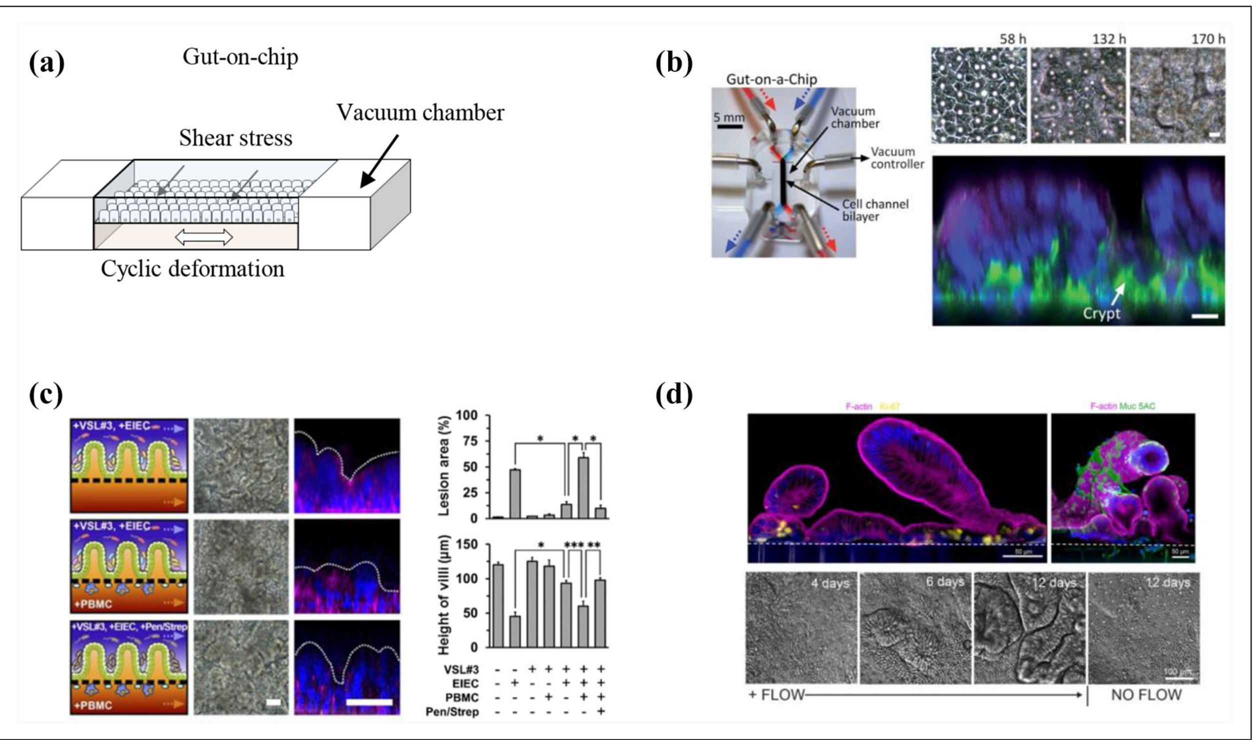

Figure 5. Development of the gut-on-chip system. (a) Schematic representing the gut-on-chip device. (b) Photograph of the gut-on-chip and bright-field images illustrating the spontaneous formation of villi-like structures by Caco-2 cells in the presence of flow and cyclic strain. Bottom panel: fluorescence image of vertical cross section of the epithelium showing the 3D organization (adapted from Chung et al.[107]). (c) This system was used to mimic inflammatory disease. Left panel: morphological analysis of villi-like structures under different conditions; columns show from left to right, schematics, phase contrast and fluorescence images of villi. Right panel: quantification of villi injury (adapted from Kim et al.[109]). (d) A gut-on-chip was next developed with primary intestinal cells obtained from dissociated organoids. Upper panel shows fluorescence images (cross section and 3D reconstruction) of villi structures with proliferative cells (Ki67) close to the membrane and differentiated cells (Muc 5AC) in villi. Bottom panel: bright-field images of cell culture on chip under flow for up to 12 days, compared to 12 days in absence of flow (adapted from Kim et al.[111]).

a basal vascular zone (Figure 5(d)).[113] Similarly, dynamic culture conditions, and specifically the fluid flow, were sufficient to induce villi formation and self-organization in crypt/villi-like domains.[112,113] The mechanism proposed to explain villi morphogenesis is the basal secretion of the Wnt antagonist, Dickkopf-1 (Dkk1) by epithelial cells. Under flow, Dkk1 is washed off, which allows Wnt signaling and initiation of morphogenesis. In opposition, under static conditions, the secreted antagonist accumulates in the basal chamber and inhibits villi formation.[114] In summary, these microfluidic chips highlight the importance of dynamic forces in intestinal development and homeostasis and appear as critical elements to incorporate in *in vitro* systems to reproduce the intestinal tissue more accurately. They also represent an ideal tool to study tissue interaction and nutrient or drug absorption.

Conclusion and future development

The advent of tissue engineering and the contribution of microfabrication processes to culture models has enabled the development of more physiological and relevant culture systems that reproduce key features of tissues. These bioengineered models have the potential to offer relatively simple, reproducible and easily manageable platforms allowing fine control of critical culture parameters, which is not the case with other *in vitro* systems or *in vivo* models.

A component that has not been fully addressed yet in intestinal epithelial models is the crucial role of the mesenchyme. Indeed, epithelial cells are surrounded and influenced by the stroma, which is composed of numerous mesenchymal cells, including fibroblasts, pericytes, smooth muscle cells, immune cells or even nervous cells.[115] The integrity of the epithelium is dependent on this mesenchyme, mainly via the secretion of various signaling molecules, notably Wnt ligands.[116,117] In addition to these biochemical signals, the mesenchyme also provides physical support by secreting ECM, such as laminin, collagen and fibronectin, whose composition changes along the crypt-villus axis.[3]

The rapid progression of this field, in term of fabrication, technology and cellular biology has opened new routes toward models recapitulating all the features of the tissue in one system. Such "all-in-one" systems may be constituted of 3D scaffolds integrated into microphysiological systems that would allow controlling the flow in the basal and luminal

compartments and reproducing the dynamic peristaltic forces. To reproduce the heterogeneity and complexity of the intestinal epithelium, primary human intestinal stem cells, or human iPSCs could be used. This approach would provide a simultaneous control of the mechanical cues, flow distribution and biochemical gradients in a 3D engineered model populated with relevant cellular types.

Bioengineered systems are also very promising tools for personalized medicine approaches.[118] Organoids can be used, but the lack of standardization and quality control of this model limits their use in biomedical applications. However, development of personalized intestine chip containing epithelial, mesenchymal, immune cells and microbiome from the same patient will offer powerful models to predict patient-specific drug response. In the context of personalized and regenerative medicine, iPSCs appear as promising tools for the generation of patient-specific intestinal tissue to study human disease and develop specific therapeutic strategies. Moreover, with the advent of the CRISPR-Cas9 mediated genome editing technology, it is now possible to genetically engineer iPSC or directly organoids[119] to mimic disease or cancer, but also to correct disease-causing mutations in patients' tissues, opening new roads for regenerative and therapeutic medicine. Finally, the development of new bioprinting approaches, such as the BATE technology, allowing the generation of macroscale tissues could ultimately provide artificial organs for drug screening or even replacement organs for regenerative therapy.

In conclusion, bioengineered systems represent a promising tool in regenerative and personalized medicine but also for the study of fundamental biological or biophysical mechanisms, as well as for the development and screening of new therapeutics molecules. This research field is still in its early stages and exponential developments in this type of technology will happen in the coming years.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work is supported by funds from the Fondation pour la Recherche Medicale (FRM Equipes DEQ20170336707) and the Institut National du Cancer (INCa PL-BIO 2020-093). It was partly supported as part of the HoliFAB project funded by the European Union's Horizon 2020 research and innovation program (grant agreement No 760927).

ORCID iDs

Justine Creff https://orcid.org/0000-0001-6101-4682
Arnaud Besson https://orcid.org/0000-0002-9599-3943

References

1. Clevers H. The intestinal crypt, a prototype stem cell compartment. *Cell* 2013; 154: 274–284.
2. Gehart H and Clevers H. Tales from the crypt: new insights into intestinal stem cells. *Nat Rev Gastroenterol Hepatol* 2019; 16: 19–34.
3. Meran L, Baulies A and Li VSW. Intestinal stem cell niche: the extracellular matrix and cellular components. *Stem Cells Int* 2017; 2017: 7970385.
4. Fatehullah A, Tan SH and Barker N. Organoids as an in vitro model of human development and disease. *Nat Cell Biol* 2016; 18: 246–254.
5. Yin X, Mead BE, Safaee H, et al. Engineering stem cell organoids. *Cell Stem Cell* 2016; 18: 25–38.
6. Costa J and Ahluwalia A. Advances and current challenges in intestinal in vitro model engineering: a digest. *Front Bioeng Biotechnol* 2019; 7: 144.
7. Wang Y, Kim R, Hinman SS, et al. Bioengineered systems and designer matrices that recapitulate the intestinal stem cell niche. *Cell Mol Gastroenterol Hepatol* 2018; 5: 440–453.e1.
8. Volk N and Lacy B. Anatomy and physiology of the small bowel. *Gastrointest Endosc Clin N Am*. 2017; 27: 1–13.
9. Barker N. Adult intestinal stem cells: critical drivers of epithelial homeostasis and regeneration. *Nat Rev Mol Cell Biol* 2014; 15: 19–33.
10. Leblond CP and Walker BE. Renewal of cell populations. *Physiol Rev* 1956; 36: 255–276.
11. Round JL and Mazmanian SK. The gut microbiota shapes intestinal immune responses during health and disease. *Nat Rev Immunol* 2009; 9: 313–323.
12. Backhed F, Ley RE, Sonnenburg JL, et al. Host-bacterial mutualism in the human intestine. *Science* 2005; 307: 1915–1920.
13. Santos AJM, Lo YH, Mah AT, et al. The intestinal stem cell niche: homeostasis and adaptations. *Trends Cell Biol* 2018; 28: 1062–1078.
14. Barker N, van Es JH, Kuipers J, et al. Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* 2007; 449: 1003–1007.
15. van der Flier LG and Clevers H. Stem cells, self-renewal, and differentiation in the intestinal epithelium. *Annu Rev Physiol* 2009; 71: 241–260.
16. Mabbott NA, Donaldson DS, Ohno H, et al. Microfold (M) cells: important immunosurveillance posts in the intestinal epithelium. *Mucosal Immunol* 2013; 6: 666–677.
17. Kim YS and Ho SB. Intestinal goblet cells and mucins in health and disease: recent insights and progress. *Curr Gastroenterol Rep* 2010; 12: 319–330.
18. Worthington JJ, Reimann F and Gribble FM. Enteroendocrine cells-sensory sentinels of the intestinal environment and orchestrators of mucosal immunity. *Mucosal Immunol* 2018; 11: 3–20.
19. Gerbe F, Legraverend C and Jay P. The intestinal epithelium tuft cells: specification and function. *Cell Mol Life Sci* 2012; 69: 2907–2917.
20. Smith RJ, Rao-Bhatia A and Kim TH. Signaling and epigenetic mechanisms of intestinal stem cells and progenitors: insight into crypt homeostasis, plasticity, and niches. *Wiley Interdiscip Rev Dev Biol* 2017; 6.

21. van Es JH, Haegebarth A, Kujala P, et al. A critical role for the Wnt effector Tcf4 in adult intestinal homeostatic self-renewal. *Mol Cell Biol* 2012; 32: 1918–1927.
22. Basak O, Beumer J, Wiebrands K, et al. Induced quiescence of Lgr5+ stem cells in intestinal organoids enables differentiation of hormone-producing enteroendocrine cells. *Cell Stem Cell* 2017; 20: 177–190.e4.
23. Riccio O, van Gijn ME, Bezdek AC, et al. Loss of intestinal crypt progenitor cells owing to inactivation of both Notch1 and Notch2 is accompanied by derepression of CDK inhibitors p27Kip1 and p57Kip2. *EMBO Rep* 2008; 9: 377–383.
24. He XC, Zhang J, Tong WG, et al. BMP signaling inhibits intestinal stem cell self-renewal through suppression of Wnt-beta-catenin signaling. *Nat Genet* 2004; 36: 1117–1121.
25. Teller IC and Beaulieu JF. Interactions between laminin and epithelial cells in intestinal health and disease. *Expert Rev Mol Med* 2001; 3: 1–18.
26. Beaulieu JF. Differential expression of the VLA family of integrins along the crypt-villus axis in the human small intestine. *J Cell Sci* 1992; 102(Pt 3): 427–436.
27. Johnson LA, Rodansky ES, Sauder KL, et al. Matrix stiffness corresponding to strictured bowel induces a fibrogenic response in human colonic fibroblasts. *Inflamm Bowel Dis* 2013; 19: 891–903.
28. Petrey AC and de la Motte CA. The extracellular matrix in IBD: a dynamic mediator of inflammation. *Curr Opin Gastroenterol* 2017; 33: 234–238.
29. Dutton JS, Hinman SS, Kim R, et al. Primary cell-derived intestinal models: recapitulating physiology. *Trends Biotechnol* 2019; 37: 744–760.
30. de Souza HS and Fiocchi C. Immunopathogenesis of IBD: current state of the art. *Nat Rev Gastroenterol Hepatol* 2016; 13: 13–27.
31. Ye Y, Pang Z, Chen W, et al. The epidemiology and risk factors of inflammatory bowel disease. *Int J Clin Exp Med* 2015; 8: 22529–22542.
32. Jairath V and Feagan BG. Global burden of inflammatory bowel disease. *Lancet Gastroenterol Hepatol* 2020; 5: 2–3.
33. Collaborators GBDIBD. The global, regional, and national burden of inflammatory bowel disease in 195 countries and territories, 1990-2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet Gastroenterol Hepatol* 2020; 5: 17–30.
34. Ruffolo C, Scarpa M and Bassi N. Infliximab, azathioprine, or combination therapy for Crohn's disease. *N Engl J Med* 2010; 363: 1086–1087; author reply 7–8.
35. Frolkis AD, Dykeman J, Negron ME, et al. Risk of surgery for inflammatory bowel diseases has decreased over time: a systematic review and meta-analysis of population-based studies. *Gastroenterology* 2013; 145: 996–1006.
36. Yoo JH and Donowitz M. Intestinal enteroids/organoids: a novel platform for drug discovery in inflammatory bowel diseases. *World J Gastroenterol* 2019; 25: 4125–4147.
37. Zhou Q, Shen ZF, Wu BS, et al. Risk of colorectal cancer in ulcerative colitis patients: a systematic review and meta-analysis. *Gastroenterol Res Pract* 2019; 2019: 5363261.
38. Mattar MC, Lough D, Pishvaian MJ, et al. Current management of inflammatory bowel disease and colorectal cancer. *Gastrointest Cancer Res* 2011; 4: 53–61.
39. Dekker E, Tanis PJ, Vleugels JLA, et al. Colorectal cancer. *Lancet* 2019; 394: 1467–1480.
40. Sato T, Vries RG, Snippert HJ, et al. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* 2009; 459: 262–265.
41. Meacham CE and Morrison SJ. Tumour heterogeneity and cancer cell plasticity. *Nature* 2013; 501: 328–337.
42. Mak IW, Evaniew N and Ghert M. Lost in translation: animal models and clinical trials in cancer treatment. *Am J Transl Res* 2014; 6: 114–118.
43. Guidelines for the treatment of animals in behavioural research and teaching. *Anim Behav* 2000; 59: 253–257.
44. Mattei G, Giusti S and Ahluwalia A. Design criteria for generating physiologically relevant in vitro models in bioreactors. *Processes* 2014; 2: 548–569.
45. Simon-Assmann P, Turck N, Sidhoum-Jenny M, et al. In vitro models of intestinal epithelial cell differentiation. *Cell Biol Toxicol* 2007; 23: 241–256.
46. Hidalgo IJ, Raub TJ and Borchardt RT. Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology* 1989; 96: 736–749.
47. Antunes F, Andrade F, Araujo F, et al. Establishment of a triple co-culture in vitro cell models to study intestinal absorption of peptide drugs. *Eur J Pharm Biopharm* 2013; 83: 427–435.
48. Walter E, Janich S, Roessler BJ, et al. HT29-MTX/Caco-2 cocultures as an in vitro model for the intestinal epithelium: in vitro–in vivo correlation with permeability data from rats and humans. *J Pharm Sci* 1996; 85: 1070–1076.
49. Pontier C, Pachot J, Botham R, et al. HT29-MTX and Caco-2/TC7 monolayers as predictive models for human intestinal absorption: role of the mucus layer. *J Pharm Sci* 2001; 90: 1608–1619.
50. Ahmed D, Eide PW, Eilertsen IA, et al. Epigenetic and genetic features of 24 colon cancer cell lines. *Oncogenesis* 2013; 2: e71.
51. Lancaster MA and Knoblich JA. Organogenesis in a dish: modeling development and disease using organoid technologies. *Science* 2014; 345: 1247125.
52. Sato T and Clevers H. Growing self-organizing mini-guts from a single intestinal stem cell: mechanism and applications. *Science* 2013; 340: 1190–1194.
53. Ho BX, Pek NMQ and Soh BS. Disease modeling using 3D organoids derived from human induced pluripotent stem cells. *Int J Mol Sci* 2018; 19: 936.
54. Hill DR and Spence JR. Gastrointestinal organoids: understanding the molecular basis of the host-microbe interface. *Cell Mol Gastroenterol Hepatol* 2017; 3: 138–149.
55. Spence JR, Mayhew CN, Rankin SA, et al. Directed differentiation of human pluripotent stem cells into intestinal tissue in vitro. *Nature* 2011; 470: 105–109.
56. Finkbeiner SR and Spence JR. A gutsy task: generating intestinal tissue from human pluripotent stem cells. *Dig Dis Sci* 2013; 58: 1176–1184.
57. Jung KB, Lee H, Son YS, et al. Interleukin-2 induces the in vitro maturation of human pluripotent stem cell-derived intestinal organoids. *Nat Commun* 2018; 9: 3039.
58. Watson CL, Mahe MM, Munera J, et al. An in vivo model of human small intestine using pluripotent stem cells. *Nat Med* 2014; 20: 1310–1314.

59. Wilson SS, Tocchi A, Holly MK, et al. A small intestinal organoid model of non-invasive enteric pathogen-epithelial cell interactions. *Mucosal Immunol* 2015; 8: 352–361.
60. Moon C, VanDussen KL, Miyoshi H, et al. Development of a primary mouse intestinal epithelial cell monolayer culture system to evaluate factors that modulate IgA transcytosis. *Mucosal Immunol* 2014; 7: 818–828.
61. Wang Y, DiSalvo M, Gunasekara DB, et al. Self-renewing monolayer of primary colonic or rectal epithelial cells. *Cell Mol Gastroenterol Hepatol* 2017; 4: 165–182.e7.
62. Thorne CA, Chen IW, Sanman LE, et al. Enteroid monolayers reveal an autonomous WNT and BMP circuit controlling intestinal epithelial growth and organization. *Dev Cell* 2018; 44: 624–633.e4.
63. Altay G, Larranaga E, Tosi S, et al. Self-organized intestinal epithelial monolayers in crypt and villus-like domains show effective barrier function. *Sci Rep* 2019; 9: 10140.
64. Rossi G, Manfrin A and Lutolf MP. Progress and potential in organoid research. *Nat Rev Genet* 2018; 19: 671–687.
65. Edington CD, Chen WLK, Geishecker E, et al. Interconnected microphysiological systems for quantitative biology and pharmacology studies. *Sci Rep* 2018; 8: 4530.
66. Jabaji Z, Sears CM, Brinkley GJ, et al. Use of collagen gel as an alternative extracellular matrix for the in vitro and in vivo growth of murine small intestinal epithelium. *Tissue Eng Part C Methods* 2013; 19: 961–969.
67. Sachs N, Tsukamoto Y, Kujala P, et al. Intestinal epithelial organoids fuse to form self-organizing tubes in floating collagen gels. *Development* 2017; 144: 1107–1112.
68. Ruedinger F, Lavrentieva A, Blume C, et al. Hydrogels for 3D mammalian cell culture: a starting guide for laboratory practice. *Appl Microbiol Biotechnol* 2015; 99: 623–636.
69. Blondel D and Lutolf MP. Bioinspired hydrogels for 3D organoid culture. *Chimia (Aarau)* 2019; 73: 81–85.
70. Verhulsel M, Vignes M, Descroix S, et al. A review of microfabrication and hydrogel engineering for micro-organs on chips. *Biomaterials* 2014; 35: 1816–1832.
71. Zhu J. Bioactive modification of poly(ethylene glycol) hydrogels for tissue engineering. *Biomaterials* 2010; 31: 4639–4656.
72. Phelps EA, Landazuri N, Thule PM, et al. Bioartificial matrices for therapeutic vascularization. *Proc Natl Acad Sci USA* 2010; 107: 3323–3328.
73. Hern DL and Hubbell JA. Incorporation of adhesion peptides into nonadhesive hydrogels useful for tissue resurfacing. *J Biomed Mater Res* 1998; 39: 266–276.
74. Gobin AS and West JL. Effects of epidermal growth factor on fibroblast migration through biomimetic hydrogels. *Biotechnol Prog* 2003; 19: 1781–1785.
75. Gjorevski N and Lutolf MP. Synthesis and characterization of well-defined hydrogel matrices and their application to intestinal stem cell and organoid culture. *Nat Protoc* 2017; 12: 2263–2274.
76. Gjorevski N, Sachs N, Manfrin A, et al. Designer matrices for intestinal stem cell and organoid culture. *Nature* 2016; 539: 560–564.
77. Cruz-Acuna R, Quiros M, Farkas AE, et al. Synthetic hydrogels for human intestinal organoid generation and colonic wound repair. *Nat Cell Biol* 2017; 19: 1326–1335.
78. Cruz-Acuna R, Quiros M, Huang S, et al. PEG-4MAL hydrogels for human organoid generation, culture, and in vivo delivery. *Nat Protoc* 2018; 13: 2102–2119.
79. Huang G, Li F, Zhao X, et al. Functional and biomimetic materials for engineering of the three-dimensional cell microenvironment. *Chem Rev* 2017; 117: 12764–12850.
80. Hinman SS, Kim R, Wang Y, et al. Microphysiological system design: simplicity is elegance. *Curr Opin Biomed Eng* 2020; 13: 94–102.
81. Wang Y, Gunasekara DB, Reed MI, et al. A microengineered collagen scaffold for generating a polarized crypt-villus architecture of human small intestinal epithelium. *Biomaterials* 2017; 128: 44–55.
82. Castano AG, Garcia-Diaz M, Torras N, et al. Dynamic photopolymerization produces complex microstructures on hydrogels in a moldless approach to generate a 3D intestinal tissue model. *Biofabrication* 2019; 11: 025007.
83. Huh D, Hamilton GA and Ingber DE. From 3D cell culture to organs-on-chips. *Trends Cell Biol* 2011; 21: 745–754.
84. Cha C, Piraino F and Khademhosseini A. Microfabrication technology in tissue engineering. In: Clemens A, Van B and Jan D (eds) *Tissue engineering*. London: Elsevier, 2014, pp.283–310.
85. Weibel DB, Diluzio WR and Whitesides GM. Microfabrication meets microbiology. *Nat Rev Microbiol* 2007; 5: 209–218.
86. Gross BC, Erkal JL, Lockwood SY, et al. Evaluation of 3D printing and its potential impact on biotechnology and the chemical sciences. *Anal Chem* 2014; 86: 3240–3253.
87. Murphy SV and Atala A. 3D bioprinting of tissues and organs. *Nat Biotechnol* 2014; 32: 773–785.
88. Mandrycky C, Wang Z, Kim K, et al. 3D bioprinting for engineering complex tissues. *Biotechnol Adv* 2016; 34: 422–434.
89. Gao B, Yang Q, Zhao X, et al. 4D bioprinting for biomedical applications. *Trends Biotechnol* 2016; 34: 746–756.
90. Sung JH, Yu J, Luo D, et al. Microscale 3-D hydrogel scaffold for biomimetic gastrointestinal (GI) tract model. *Lab Chip* 2011; 11: 389–392.
91. Yu J, Peng S, Luo D, et al. In vitro 3D human small intestinal villous model for drug permeability determination. *Biotechnol Bioeng* 2012; 109: 2173–2178.
92. Kim SH, Chi M, Yi B, et al. Three-dimensional intestinal villi epithelium enhances protection of human intestinal cells from bacterial infection by inducing mucin expression. *Integr Biol (Camb)* 2014; 6: 1122–1131.
93. Costello CM, Hongpeng J, Shaffiey S, et al. Synthetic small intestinal scaffolds for improved studies of intestinal differentiation. *Biotechnol Bioeng* 2014; 111: 1222–1232.
94. Costello CM, Sorna RM, Goh YL, et al. 3-D intestinal scaffolds for evaluating the therapeutic potential of probiotics. *Mol Pharm* 2014; 11: 2030–2039.
95. Altay G, Tosi S, Garcia-Diaz M, et al. Imaging the cell morphological response to 3D topography and curvature in engineered intestinal tissues. *Front Bioeng Biotechnol* 2020; 8: 294.
96. Wang L, Murthy SK, Fowle WH, et al. Influence of micro-well biomimetic topography on intestinal epithelial Caco-2 cell phenotype. *Biomaterials* 2009; 30: 6825–6834.
97. Wang L, Murthy SK, Barabino GA, et al. Synergic effects of crypt-like topography and ECM proteins on intestinal cell behavior in collagen based membranes. *Biomaterials* 2010; 31: 7586–7598.
98. Madden LR, Nguyen TV, Garcia-Mojica S, et al. Bioprinted 3D primary human intestinal tissues model

aspects of native physiology and ADME/Tox functions. *iScience* 2018; 2: 156–167.
99. Kim W and Kim G. Intestinal villi model with blood capillaries fabricated using collagen-based bioink and dual-cell-printing process. *ACS Appl Mater Interfaces* 2018; 10: 41185–41196.
100. Kim W and Kim GH. An innovative cell-printed microscale collagen model for mimicking intestinal villus epithelium. *Chem Eng J* 2018; 334: 2308–2318.
101. Creff J, Courson R, Mangeat T, et al. Fabrication of 3D scaffolds reproducing intestinal epithelium topography by high-resolution 3D stereolithography. *Biomaterials* 2019; 221: 119404.
102. Nikolaev M, Mitrofanova O, Broguiere N, et al. Homeostatic mini-intestines through scaffold-guided organoid morphogenesis. *Nature* 2020; 585: 574–578.
103. Brassard JA, Nikolaev M, Hubscher T, et al. Recapitulating macro-scale tissue self-organization through organoid bioprinting. *Nat Mater*. Epub ahead of print September 2020. DOI: 10.1038/s41563-020-00803-5.
104. Bhatia SN and Ingber DE. Microfluidic organs-on-chips. *Nat Biotechnol* 2014; 32: 760–772.
105. Whitesides GM. The origins and the future of microfluidics. *Nature* 2006; 442: 368–373.
106. Li Jeon N, Baskaran H, Dertinger SK, et al. Neutrophil chemotaxis in linear and complex gradients of interleukin-8 formed in a microfabricated device. *Nat Biotechnol* 2002; 20: 826–830.
107. Chung BG, Flanagan LA, Rhee SW, et al. Human neural stem cell growth and differentiation in a gradient-generating microfluidic device. *Lab Chip* 2005; 5: 401–406.
108. Lang S, von Philipsborn AC, Bernard A, et al. Growth cone response to ephrin gradients produced by microfluidic networks. *Anal Bioanal Chem* 2008; 390: 809–816.
109. Kim HJ, Huh D, Hamilton G, et al. Human gut-on-a-chip inhabited by microbial flora that experiences intestinal peristalsis-like motions and flow. *Lab Chip* 2012; 12: 2165–2174.
110. Kim HJ and Ingber DE. Gut-on-a-Chip microenvironment induces human intestinal cells to undergo villus differentiation. *Integr Biol (Camb)* 2013; 5: 1130–1140.
111. Kim HJ, Li H, Collins JJ, et al. Contributions of microbiome and mechanical deformation to intestinal bacterial overgrowth and inflammation in a human gut-on-a-chip. *Proc Natl Acad Sci USA* 2016; 113: E7–E15.
112. Workman MJ, Gleeson JP, Troisi EJ, et al. Enhanced utilization of induced pluripotent stem cell-derived human intestinal organoids using microengineered chips. *Cell Mol Gastroenterol Hepatol* 2018; 5: 669–677.e2.
113. Kasendra M, Tovaglieri A, Sontheimer-Phelps A, et al. Development of a primary human Small Intestine-on-a-Chip using biopsy-derived organoids. *Sci Rep* 2018; 8: 2871.
114. Shin W, Hinojosa CD, Ingber DE, et al. Human intestinal morphogenesis controlled by transepithelial morphogen gradient and flow-dependent physical cues in a microengineered gut-on-a-chip. *iScience* 2019; 15: 391–406.
115. Powell DW, Pinchuk IV, Saada JI, et al. Mesenchymal cells of the intestinal lamina propria. *Annu Rev Physiol* 2011; 73: 213–237.
116. Kabiri Z, Greicius G, Madan B, et al. Stroma provides an intestinal stem cell niche in the absence of epithelial Wnts. *Development* 2014; 141: 2206–2215.
117. Gregorieff A, Pinto D, Begthel H, et al. Expression pattern of Wnt signaling components in the adult intestine. *Gastroenterology* 2005; 129: 626–638.
118. Huang J, Ren Y, Wu X, et al. Gut bioengineering promotes gut repair and pharmaceutical research: a review. *J Tissue Eng* 2019; 10: 2041731419839846.
119. Fujii M, Clevers H and Sato T. Modeling human digestive diseases with CRISPR-Cas9-modified Organoids. *Gastroenterology* 2019; 156: 562–576.