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Three dimensional human small intestine models for ADME-Tox studies

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In vitro **human small intestine models play a crucial part in preclinical drug development. Although conventional 2D systems possess many advantages, such as facile accessibility and high-throughput capability, they can also provide misleading results due to their relatively poor recapitulation of *in vivo* physiology. Significant progress has recently been made in developing 3D human small intestine models, suggesting that more-reliable preclinical results could be obtained by recreating the 3D intestinal microenvironment *in vitro*. Although there are still many challenges, 3D human small intestine models have the potential to facilitate drug screening and drug development.**

Introduction

Oral delivery is considered as the most preferable and convenient route among various drug administration methods. Before reaching the systemic circulation, orally administrated drugs are absorbed by the small intestine epithelium and undergo first-pass metabolism. By utilizing porous membranes, such as track-etched membranes (membranes with cylindrical pores produced by using etching ion tracks) [1], for culturing confluent Caco-2 cell monolayers, a 2D *in vitro* cell-based human small intestine model was developed over 20 years ago [2]. This model has been widely used for studying oral drug absorption [3], intestinal transporters [4], intestinal first-pass metabolism [5,6] and toxicity [7,8]. To make the model amenable to high-throughput intestinal permeability measurement, multiple approaches have been adopted that include reduction of Caco-2 culture time [9], use of fast-growing cell lines such as Madin–Darby canine kidney (MDCK) [10], integration of robotic liquid handling systems [11] and development of automated liquid chromatography tandem mass spectrometry (LC–MS/MS) systems [12]. In the FDA's Biopharmaceutics Classification System (BCS) guidance (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070246.pdf), Caco-2 and MDCK 2D models have been suggested as alternatives to animal or human models for evaluating drug intestinal permeability [13]. *In silico* drug permeability prediction models also rely on the 2D *in vitro* small intestine model for development and validation [14]. Overall, the 2D *in vitro* small intestine model has been widely recognized as a very useful screening tool that has not only greatly reduced animal use but also facilitated the drug discovery and preclinical development processes.

However, the pharmaceutical industry is still suffering high attrition rates, with Phase I clinical drug candidates having less than 10% chance of making it to the market [15]. Because it has been estimated that the average time from synthesis of a compound to Phase I clinical trial for self-originated drugs is 52 months [16], more-reliable preclinical results are needed to increase the efficiency of the drug development process. Although an important preclinical screening tool, the 2D *in vitro* small intestine model has been known to give false-positive or false-negative results owing to lack of physiological relevance [17]. For example, the 2D Caco-2 model has been suggested as an excellent model for predicting intestinal permeability of rapidly absorbed drugs that rely on the passive transcellular pathway for absorption. However, for slowly absorbed drugs utilizing the paracellular pathway and actively transported drugs utilizing carrier-mediated pathways, the model generally does not predict intestinal permeability accurately [3]. Although many approaches have been adapted to diminish the gap between the conventional 2D small intestine model and human small intestine, there is still a growing need for a better *in vitro* model with

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1359-6446/06/$ - see front matter © 2014 Published by Elsevier Ltd. http://dx.doi.org/10.1016/j.drudis.2014.05.003

greater complexity that represents *in vivo* characteristics of native intestine [3,14].

By utilizing microfabrication, microfluidics, biomaterials, tissue engineering and stem cell techniques, various 3D human small intestine models have been developed in the past five years. Aiming to create a more realistic human small intestine microenvironment *in vitro*, physiological 3D features have been integrated in these models, enabling significant improvements over the conventional 2D model. Although for practical screening use, many aspects of 3D models are still underdeveloped, preliminary results from 3D models suggest that robust preclinical results could be obtained when *in vivo* features are mimicked in a more authentic fashion by *in vitro* models. In this review, we summarize recently developed 3D human small intestine models. The advantages and limitations of 3D models as well as their potential impact on ADME-Tox screening are discussed.

The gap between human small intestine and conventional *in vitro* human small intestine model

A brief overview of human small intestine physiology

Human small intestine, which includes the duodenum, jejunum and ileum, is a convoluted tube that is, on average, 2.5 cm in diameter and 3 m long (in a living person) [18]. To gain the maximum efficiency of absorption and digestion, the human small intestine possesses highly complex 3D microenvironments (Fig. 1a). The small intestine mucosa and submucosa form circular folds (also called *valvulae conniventes* or Kerckring's folds) that protrude into the lumen transversely and increase the retention time of chyme. These permanent ridges, which are about 1 cm tall, begin to appear near the proximal portion of the duodenum and disappear nearly completely in the distal ileum [18,19]. The surface of these circular folds also possesses finger-like protrusions called intestinal villi. Each villus is about 0.5–1.0 mm high, and there are 20–40 villi per mm^2 [18,20] (Fig. 1c). The presence of circular folds and villi greatly increases the absorption surface area. Between villi, the small intestine mucosa contains deep tubular pits called intestinal crypts. Residing inside the crypt, small intestine stem cells can differentiate into four different intestinal epithelial cell types, which are the enterocyte (absorptive cell), goblet, enteroendocrine and Paneth cells. Although Paneth cells reside within the stem cell niche at the bottom of the crypt, the other three types of intestinal epithelial cells migrate toward the tip of the villus, forming a columnar epithelial monolayer that covers the intestinal mucosa. The intestinal blood flow in close proximity to the basolateral surface and the chyme movement formed by intestinal peristaltic waves at the apical surface provide a dynamic environment for the epithelial monolayer. The crypt and villus structures on the mucosa surface also generate chemical gradients that probably affect the epithelial cell physiology. The small intestinal lumen also has a large population of commensal bacteria with a density increasing from 10^3 organisms per ml of luminal contents in duodenum to 10^9 per ml of luminal contents in the distal ileum [21]. Within this dynamic and complex environment, the small intestine epithelium has the most vigorous renewal process in adult epithelial tissues, with the epithelial monolayer regenerating every 4–5 days.

Conventional in vitro *models lack small intestine 3D structural features*

In the conventional in vitro small intestine model, track-etched membranes are typically used to support the monolayer culture [1] (Fig. 1b). These porous membranes have pore sizes ranging from 0.4 to 8.0 μm and are commercially available as cell culture inserts with six-well, 12-well and 24-well formats. Various extracellular matrix proteins can be coated on the membrane surface, and the epithelial cell monolayer is cultured on top of the membrane to mimic the small intestine epithelial barrier. Compared with the human small intestine, the conventional *in vitro* model suffers three major drawbacks. First, because track-etched membranes have flat surfaces, conventional *in vitro* models lack small intestine 3D structural features. It has been well recognized that, compared with 2D culture, cells behave in a more authentic manner within a 3D culture microenvironment [22]. The small intestine 3D structures not only increase the absorptive surface area but also create biochemical and physical signal gradients that probably affect cell function, cell proliferation and cell differentiation. For example, increasing expression gradients of digestive enzymes such as alkaline phosphatases, disaccharidases and dipeptidases have been described along the crypt–villus axis in rat [23]. Similar expression gradients of phase I and phase II drug-metabolizing enzymes including cytochrome P450 (CYP) 1A1 (CYP1A1), CYP2B1, UDP-glucuronosyltransferase and glutathione-S-transferase have also been suggested in rat [24,25]. Moreover, increasing expression patterns along the crypt–villus axis have been observed for drug transporters called organic anion-transporting polypeptide (OATP)1A2 and P-glycoprotein [also known as multi-drug resistance protein (MDR1)] in human as well as MRP2 in rat [26,27]. The paracellular pore size of rat epithelial monolayer has been found to decrease from the crypt to the top of villus [28]. Various distribution patterns of epithelial basement membrane proteins have also been indicated along the crypt–villus axis in human [29]. Epithelial monolayers cultured in conventional *in vitro* models lacking 3D microenvironment inherently cannot represent these physiological features, which could lead to false predictions in drug ADME-Tox properties. Hence, it is crucial to integrate small intestine 3D features into the *in vitro* model. Heterogeneous expression of drug-metabolizing enzymes and drug transporters has also been implied on a macroscopic scale, along the length of small intestine in human. Total CYP content and CYP3A4 activity were found to increase slightly along the length of the duodenum before decreasing significantly toward the ileum [30]. The expression of drug transporters P-glycoprotein, breast cancer resistance protein (BCRP) and carnitine/organic cation transporter 2 (OCTN2) increased significantly from duodenum to ileum, whereas opposite expression gradients were found for monocarboxylate transporter 1 (MCT1) [31]. Current *in vitro* intestinal models are not able to capture this macroscopic variability in enzyme and transporter expression.

Conventional in vitro *models lack relevant small intestine epithelial cell types*

In conventional intestinal cell culture models, human carcinoma cell line Caco-2 and HT-29 MTX have been the main intestinal cells cultured to represent small intestine enterocytes and goblet cells, respectively [32]. Caco-2 cells have been induced to differentiate into M cells by culturing with murine Peyer's patch

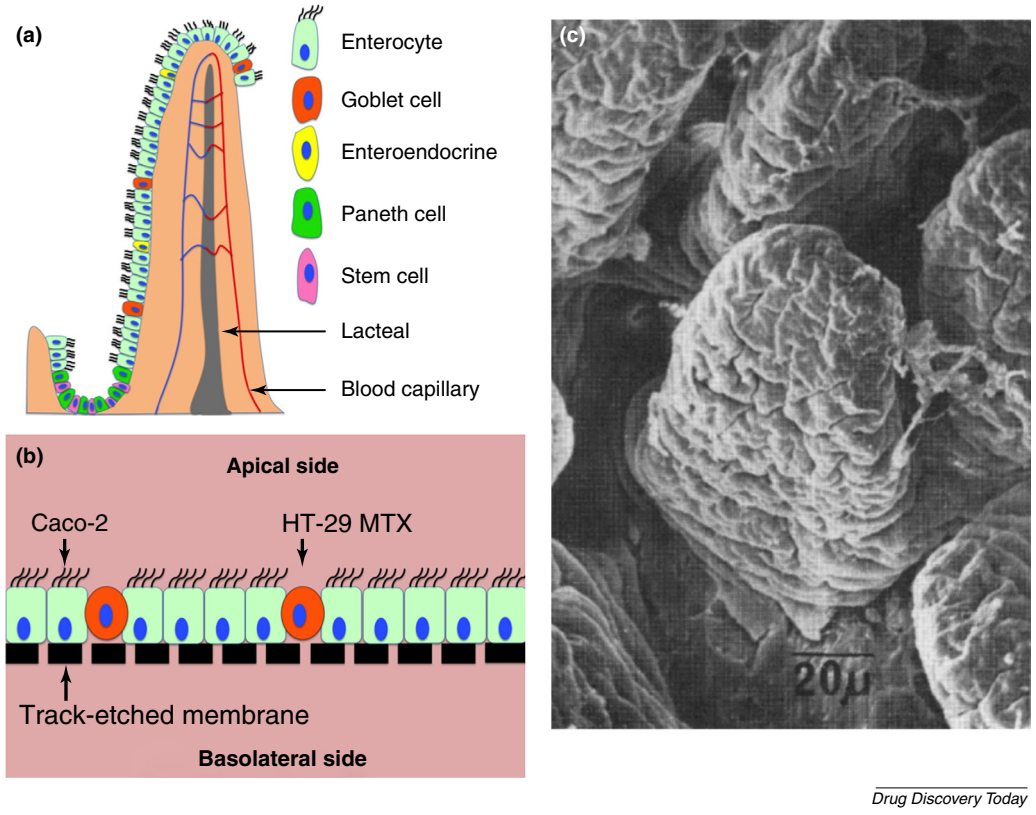

FIGURE 1

(a) Illustration of human small intestine epithelium on crypt–villus axis. **(b)** A schematic of conventional 2D small intestine model. Caco-2 and HT-29 MTX are cultured on the track-etched membrane. **(c)** A scanning electron microscope image of human small intestine villi. Reproduced, with permission, from [20].

lymphocytes or human Raji B cells [8,33]. Only mouse carcinoma cell lines have been developed to simulate *enteroendocrine cells* [34], and there is currently no Paneth cell line available for *in vitro* studies. Even for the most frequently used cell line Caco-2, which presents many comparable characteristics to small intestine enterocytes, significant differences have been found between the two. For instance, although Caco-2 cell monolayers and human small intestine epithelium have similar paracellular pore sizes, the porosity of Caco-2 monolayer is eight-times smaller than that of human small intestine epithelium, which is considered as one of the reasons for poor drug paracellular permeability correlation between conventional *in vitro* models and human small intestine [35]. Also, studies have suggested that significant differences in gene expression profiles between Caco-2 cells and human duodenum result in variation of drug transporters and drug-metabolizing enzymes between *in vitro* and *in vivo* systems, which affect drug metabolism and carrier-mediated drug transport [36]. Therefore, more-relevant cells are needed to improve the *in vitro* model.

Conventional in vitro *models lack a dynamic culture environment*

Although there has been pioneering work carried out incorporating perfusion in intestinal cell culture, most conventional *in vitro* models still lack a dynamic culture environment [37]. In standard *in vitro* intestinal cell culture, an epithelial monolayer is cultured under static conditions, and cell medium is changed every two days [38]. Under such conditions, Caco-2 monolayers take 21 days to become fully differentiated. The co-culture of commensal bacteria and epithelial monolayer can only be sustained for several hours [39]. Thus, a dynamic perfusion system on apical and basolateral sides is desirable to provide fresh nutrients, remove metabolic waste and incapacitate bacterial overgrowth in systems incorporating microbes, on a continuous basis.

3D small intestine models with artificial topography

With the development of microfabrication and other biomaterial fabrication techniques, biomimetic topography has been integrated into *in vitro* intestinal models to represent human small intestine 3D features. For example, human small intestine crypt-like topography has been fabricated using photolithography and transferred to poly(dimethylsiloxane) (PDMS) and collagen membrane substrates [40,41] (Fig. 2a,b). Compared with 2D flat substrates, Caco-2 cultured on PDMS substrates with crypt-like topography exhibited higher mitochondrial activity and lower alkaline phosphatase activity, similar to the cell phenotype in human intestinal crypts [40]. Transepithelial electrical resistance (TEER) values were found to be slightly lower for Caco-2 monolayers cultured on topographically modified collagen membranes relative to flat collagen controls, suggesting that crypt-like topography might affect tight junctions of the Caco-2 monolayer [41]. Human small intestine villus structures have also been fabricated by combining different microfabrication techniques. Initially, 3D printing (3DP) was employed to construct porous poly(lactic-co-glycolic acid) (PLGA) villous scaffolds [42]. However, owing to the resolution limitation of this technique, porous PLGA villous structures had considerably larger basal areas than human small

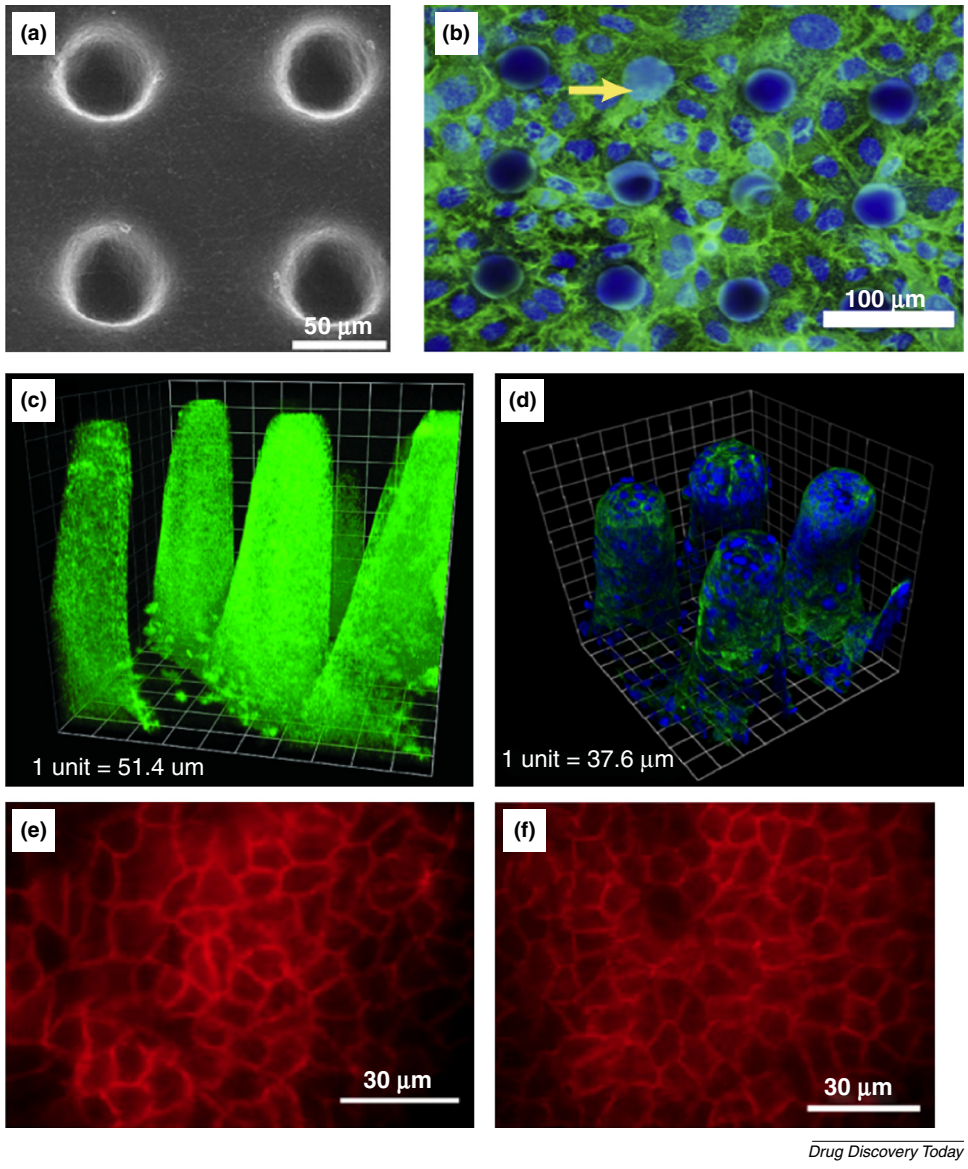

FIGURE 2

(a) Collagen membrane with crypt-like topography. Reproduced, with permission, from [41]. **(b)** Caco-2 cells cultured on poly(dimethylsiloxane) (PDMS) membrane with crypt-like topography, the yellow arrow indicates a crypt well covered by cells. Cells were stained for actin (green) and nucleic acid (blue). Reproduced, with permission, from [40]. **(c)** Confocal microscope image of a collagen villous scaffold after 3D rendering. Reproduced, with permission, from [43]. **(d)** Collagen villous scaffold covered by Caco-2 monolayer after 3D rendering. Cells were stained for actin (green) and nucleic acid (blue). Reproduced, with permission, from [44]. **(e)** Tight junction protein zonula occludens-1 (ZO-1) staining of Caco-2 cells cultured in polyethersulfone (PES) hollow fiber at day 10 [46]. **(f)** ZO-1 staining of Caco-2 cells cultured on conventional track-etched membrane at day 21. Reproduced, with permission, from [46].

intestine villi and hence could not represent dimensions and density of villi relevantly. Recently, by combining laser ablation and sacrificial molding techniques, a collagen villous scaffold was fabricated with 0.5 mm villous height and 25 villi per mm^2 density, with striking similarity to the human jejunal villi [43] (Fig. 2c,d). By using a custom-made cell culture insert kit, the collagen villous scaffold was integrated with a six-well cell culture plate to study drug absorption [44]. Caco-2 monolayers cultured on collagen villous scaffolds exhibited differentiation gradients along the villous axis, such that cells near the top of the villus were more columnar and polarized than cells near the bottom of the villus, correlating well with the *in vivo* observation that enterocytes become more differentiated when migrating from the crypt to the tip of the villus [44,45]. TEER values of Caco-2 monolayers cultured on collagen villous scaffolds were also found to be significantly lower than 2D controls, and similar to *in vivo* intestinal values. The apparent permeability coefficient (Papp) of the slowly absorbed drug atenolol measured on the collagen villous scaffold was 13-times higher than that measured using conventional 2D models, and much closer to the average permeability value from perfused human small intestines, suggesting that the 3D villous model could improve the poor permeability correlation of paracellularly transported drugs between *in vitro* models and native human small intestine [3,44]. Although the collagen scaffold did not exhibit variable transport characteristics for different small molecule drugs, the scaffold itself became a significant

transport barrier for the rapidly absorbed drug antipyrine, which is absorbed through a transcellular pathway. Hence this 3D model could not be used to test a wide range of drugs with broad permeability coefficients.

Alternatively, a polyethersulfone (PES) hollow fiber bioreactor was used to mimic the small intestine lumen 3D microenvironment [46]. Under static culture conditions, Caco-2 cells grown on the inner surface of PES hollow fibers formed cell monolayers with tight junctions and expressed brush border enzymes at high levels after 10 days, which was not observed on flat PES membrane control and conventional 2D models (Fig. 2e,f). Caco-2 monolayers on PES hollow fibers also possessed higher activity of alkaline phosphatase, γ-glutamyltransferase and P-glycoprotein than conventional 2D models. Although the diameter of PES hollow fibers used in this study (900 μm) was not relevant to the diameter of human small intestine (2.5 cm), and thus the inner surface of PES hollow fibers lacked significance to *in vivo* 3D structures, it is possible that the enclosed space created within the fiber, and thus concentration of signaling factors, facilitated cell differentiation. A similar close proximity of apical cell surfaces exists for cells on crypt and villus structures. To validate the monolayer functionality, Papp values of 16 oral drugs were measured on PES hollow fibers after 10 days of culture. Similar correlations between Papp and fraction of dose absorbed in human (Fa) were found from PES hollow fibers and conventional 2D models [46].

Another type of 3D model consists of multiple layers of relevant cell types, including a flat monolayer of Caco-2 on a gel containing dendritic cells or macrophages [47,48]. Generally, to form these models, a thicker layer of collagen protein matrix than used on conventional 2D models was coated on a cell culture insert. Although the flat collagen matrix does not capture structural features of the small intestine 3D microenvironment, the integration of immune components in the collagen matrix makes this model a useful *in vitro* tool for studying inflammatory bowel disease [47].

3D small intestine models with decellularized animal tissue

Animal intestinal tissues have been widely used for drug ADME-Tox studies incorporating the Ussing Chamber technique and the precision-cut slice model [49]. Three dimensional small intestine features have recently also been created *in vitro* by utilizing decellularized animal small intestine segments. For example, Caco-2 cells and human microvascular endothelial cells (hMECs) were cultured on the apical and basolateral sides of a decellularized porcine jejunal scaffold, respectively [50]. A custom-built bioreactor system was used to provide dynamic culture conditions, such that culture medium was perfused past both sides of the porcine jejunal scaffold continuously. Under such culture conditions, Caco-2 cells assembled 3D villus-like structures with multiple cell layers whereas hMECs formed a monolayer mimicking the endothelial barrier. Caco-2 cells cultured under these dynamic 3D culture conditions displayed significantly higher P-glycoprotein expression and dipeptidase activity than the conventional 2D model. However, P-glycoprotein efflux activity of Rhodamine 123 was found to be low under the dynamic 3D culture condition, which was speculated to be the consequence of forming multiple cell layers. The Papp values of low permeability substances fluorescein and desmopressin were measured to be significantly higher under the dynamic 3D culture condition when compared with the conventional 2D model, which also suggested that the 3D model could improve drug paracellular permeability correlation [3,50]. Additionally, a decellularized rat intestinal scaffold with villus–crypt structures and vasculature has been obtained by using detergent–enzymatic treatment, and its potential for recreating functional intestine tissue was demonstrated [51]. Because there is a strong motivation to reduce animal use in drug development, the requirement of using animal tissue makes these methods less practical for preclinical drug studies than other *in vitro* models. Native tissue has also been explored as a direct template for creating biomaterials with intestinal structure. Precise structurally biomimetic silica replicas of intestine that preserved crypt–villus structures were fabricated using a porcine intestinal segment with epithelium removed [52]. This replica could be used as a reusable mold for fabricating 3D biocompatible membranes.

3D small intestine models in microfluidic systems

Microfluidic techniques have been used in developing miniaturized *in vitro* models with high-throughput capability that also exhibit the potential for creating integrated *in vitro* systems (i.e. body-on-a-chip) predicting drug ADME-Tox properties on a whole-body scale [53–55]. A conventional 2D intestinal (Caco-2) model was integrated with a microscale cell culture analog (μCCA) system including multiple organ models to investigate drug toxicity [56]. Recently, microporous SU-8 membranes were fabricated with 3D villus-like features, with the goal of incorporating a more relevant 3D intestinal microenvironment into the μCCA system [57] (Fig. 3a). Long-term perfusion culture of Caco-2 cells on track-etched membranes was demonstrated in a microfluidic device with integrated micropumps and on-chip optical fiber sensors [58]. Immune components were also included in a microfluidic small intestine model [59]. A gut-on-a-chip device was derived from a previous microfluidic lung model with the capability to achieve perfusion flow and cyclic mechanical strain to mimic intestinal shear stress and peristaltic motions, respectively [60] (Fig. 3b). Caco-2 cells cultured under such dynamic conditions not only formed monolayers with tight junctions after 3 days of culture but were also reported to develop villous-like structures with multiple cell layers over longer times (Fig. 3c). Paracellular permeability of fluorescent dextran and aminopeptidase activity were found to be significantly higher under the dynamic culture conditions relative to conventional 2D models. Long-term co-culture of *Lactobacillus rhamnosus* GG (LGG) with Caco-2 cells was demonstrated under these dynamic conditions, with the observation that LGG actually increased Caco-2 TEER values over time. Interestingly, Caco-2 cells cultured in this gut-on-a-chip stained positively for goblet, enteroendocrine and Paneth cell markers, which suggested that the dynamic culture condition could affect cell differentiation dramatically [61]. This finding challenged the traditional understanding that Caco-2 cells only differentiate into enterocyte cell types under normal culture conditions. Mucus production and CYP3A4 enzyme activity, which are usually missing in Caco-2 monolayers, were also detected under the dynamic culture condition without any drug inductions. However, the level of CYP3A4 activity was about three orders of magnitude lower than that reported for human small intestine [30]. Overall, results obtained with the gut-on-a-chip

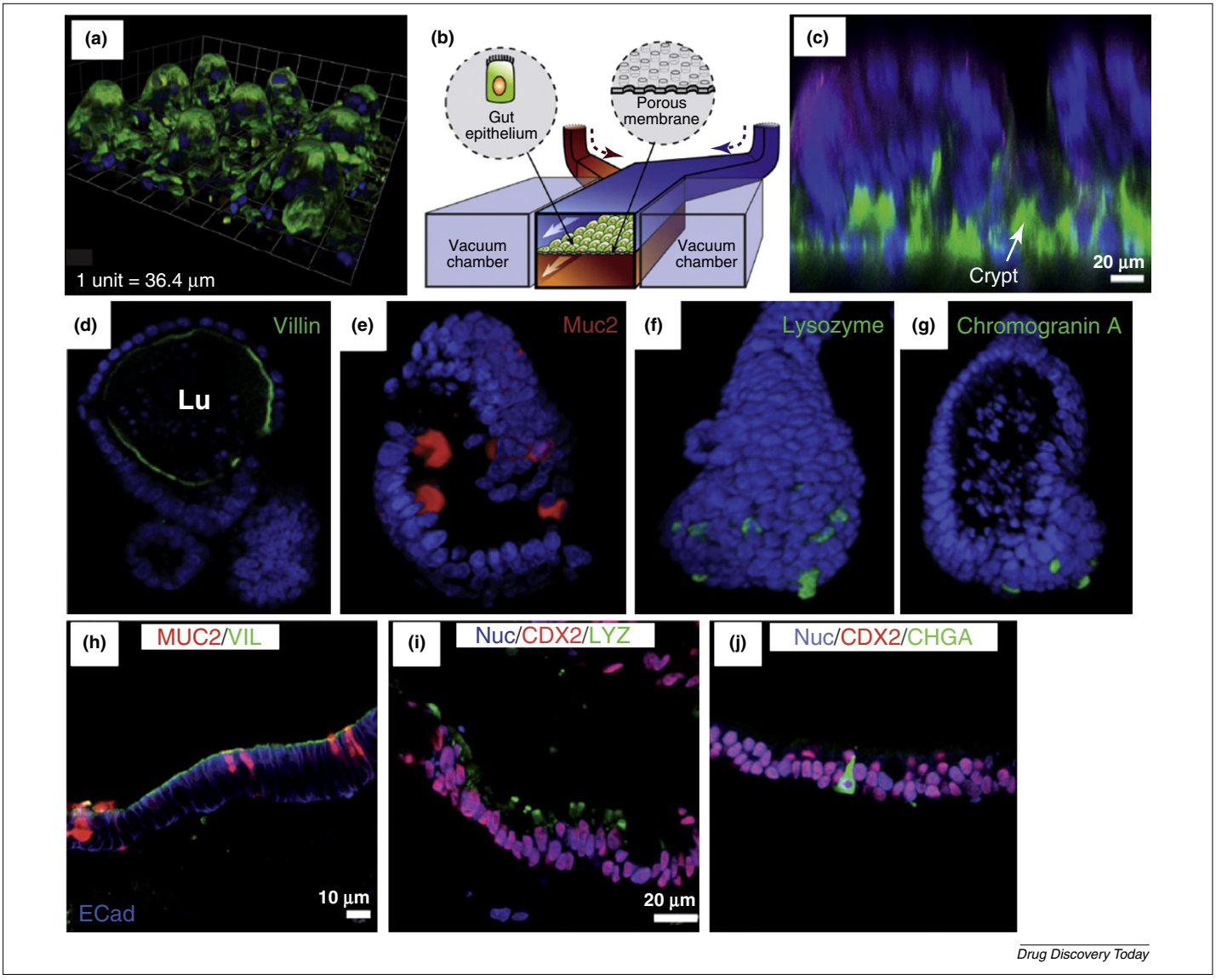

FIGURE 3

(a) Caco-2 cells grown on microporous SU-8 membranes [57]. Cells were stained for actin (green) and nucleic acid (blue). Reproduced, with permission, from [57]. **(b)** A schematic of the gut-on-a-chip device. Reproduced, with permission, from [60]. **(c)** Vertical cross section of villous-like structures [60]. Cells were stained for actin (green), nucleic acid (blue) and Muc2 (magenta). Reproduced, with permission, from [60]. **(d–g)** Single $Lgr5^+$ stem-cell-derived organoids were stained for villin [(d): green, enterocytes), Muc2 **(e)**: red, goblet cells], lysozyme **(f)**: green, Paneth cells], chromogranin A [(g): green, enteroendocrine cells] and nucleic acid (blue). Reproduced, with permission, from [63]. **(h–j)** Twenty-eight-day induced pluripotent stem cells (iPSC)-derived organoids were stained for villin [(h): green], Muc2 [(h): red], lysozyme **(i)**: green], chromogranin A [(j): green] and nucleic acid (blue). Adapted, with permission, from [65].

device suggested that introducing perfusion flow and cyclic mechanical strain could improve the physiological relevance of the *in vitro* small intestine model.

Alternative cell sources

Caco-2 cell culture models have been criticized for poor prediction of drug ADME-Tox properties, in part because of Caco-2 colon adenocarcinoma origin [3,35,36]. The absence of small intestinal stem cells and other epithelial cell types limits the relevance of physiology and functionality of cell monolayers in *in vitro* models to human small intestine. Recently, small intestine stem cells have been identified by using the Lgr5 gene marker [62]. Long-term culture based on $Lgr5^+$ stem cells demonstrated that single stem cells have the ability to differentiate into four small intestinal epithelial cell types and form organoids with crypt–villus 3D structures *in vitro* [63] (Fig. 3d–g). Long-term culture of neonatal small intestine tissue that formed organoids with four epithelial cell types has also been established in a collagen gel with an air–liquid interface [64]. Alternatively, directed differentiation of human embryonic stem cells and induced pluripotent stem cells (iPSC) into intestine-like organoids containing four intestinal cell types and crypt–villus 3D architectures has been achieved *in vitro* [65] (Fig. 3h–j). Moreover, colonic stem cell organoids have been cultured on the conventional cell culture insert forming an epithelial monolayer [66]. Although ADME-Tox properties of these intestinal organoids have not been extensively examined, it is likely that recent advances in intestinal stem cell and organoid culture can be employed to develop a more

authentic *in vitro* small intestine epithelium for drug screening studies in the future.

Concluding remarks

Three dimensional human small intestine models have demonstrated the potential to accelerate cell differentiation processes, improve paracellular permeability correlation and maintain epithelium and commensal bacteria co-culture. However, the distribution of drug transporters and metabolizing enzymes in 3D human small intestine models has not been fully investigated. Heterogeneous expressions of drug-metabolizing enzymes and drug transporters along the length of small intestine, which is crucial for ADME-Tox studies, have also been overlooked. For drug transporters like P-glycoprotein, which exhibits more than fivefold expression difference between human duodenum and ileum, it is difficult to evaluate if the transporter expression level in a particular 3D model is relevant without specifying which segment of the human small intestine the model is mimicking [31]. Future 3D models focusing on the whole small intestine instead of intestinal segments are desirable in this regard. For practical ADME-Tox studies, drugs with different properties should be tested in 3D models to validate these systems. The consequence of presenting multiple cell layers needs to be considered, because normal enterocytes only form monolayers *in vivo*. To succeed in industrial settings, the high-throughput capability, the cost and feasibility are also crucial factors for using 3D models. As further improvements and enhanced capabilities develop, 3D human small intestine models are highly likely to have a significant impact on drug preclinical development and absorption assessment.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

The paper is supported by Cooperative Agreement (Number W911NF-12-2-0039) from DARPA. R.L.C. acknowledges NSF CMMI MDSE for funding #0727984 and NSF CBET BBBE for funding #0700764. J.C.M. acknowledges the Hartwell Foundation and the Defense Threat Reduction Agency.

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