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Review

The Past, Present and Future of Intestinal *In Vitro* Cell Systems for Drug Absorption Studies

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ABSTRACT

The intestinal epithelium acts as a selective barrier for the absorption of water, nutrients and orally administered drugs. To evaluate the gastrointestinal permeability of a candidate molecule, scientists and drug developers have a multitude of cell culture models at their disposal. Static transwell cultures constitute the most extensively characterized intestinal *in vitro* system and can accurately categorize molecules into low, intermediate and high permeability compounds. However, they lack key aspects of intestinal physiology, including the cellular complexity of the intestinal epithelium, flow, mechanical strain, or interactions with intestinal mucus and microbes. To emulate these features, a variety of different culture paradigms, including microfluidic chips, organoids and intestinal slice cultures have been developed. Here, we provide an updated overview of intestinal *in vitro* cell culture systems and critically review their suitability for drug absorption studies. The available data show that these advanced culture models offer impressive possibilities for emulating intestinal complexity. However, there is a paucity of systematic absorption studies and benchmarking data and it remains unclear whether the increase in model complexity and costs translates into improved drug permeability predictions. In the absence of such data, conventional static transwell cultures remain the current gold-standard paradigm for drug absorption studies.

Introduction

Oral drug administration constitutes the most convenient route of drug delivery; however, its suitability largely depends on the oral bioavailability of the drug in questions. For novel medicines, the prediction of oral pharmacokinetics remains difficult, primarily due to the complexity of the underlying processes that include but are not limited to the permeability of the drug to pass the intestine and enter the bloodstream, as well as its metabolism in GI tract and liver.[1] Thus, strategies to accurately predict oral absorption are of tremendous importance for drug development. Over the past decades an interdisciplinary arsenal of methods has been developed that includes *in vitro* and *in vivo* models, as well as mathematical modeling, whereby the latter integrates the experimental data to provide additional physiological context. Notably, it is by now well accepted that best predictions of drug metabolism and pharmacokinetic (DMPK) parameters are achieved by an integration of data from different screening assays.

Intestinal drug absorption can occur via passive or active transcellular transport, passive paracellular transport or transcytosis (Table 1). Passive transcellular transport is a diffusion process driven by the concentration gradient of a compound and applies mostly to lipophilic compounds that easily dissolve in membranes. This mode of transport is usually not saturable, does not depend on the stereochemistry of the molecule in question and is not sensitive to transport inhibitors. In contrast, active transcellular transport is mediated by transporter proteins. As such, this process can be saturated, is stereoisomer and, in some cases, enantiomer specific and can be inhibited, which results in active transport being a common reason for drug-drug interactions.[2] Paracellular transport constitutes a passive process in which primarily small hydrophilic molecules diffuse through the tight junctions between enterocytes. It can be further subdivided into the pore pathway, which is size- and charge-selective and primarily regulated by claudins, and the leak pathway, which is less selective and whose overall permeability is regulated by ZO1, occluding and MLCK.[3] The efficiency of this pathway is assumed to be much lower than the passive transcellular pathway due to the low area of the paracellular space, suggesting that, unlike the latter, paracellular

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absorption can be saturated.[4,5] Lastly, intestinal transcytosis constitutes a receptor-mediated mechanism that utilizes the clathrin-dependent endocytosis machinery (excellently reviewed in[6]). This pathway has a very low capacity and thus can only provide a means for the delivery of highly potent drugs.

The most commonly used *in vivo* models for oral drug absorption are rats, dogs and monkeys.[7] Importantly however, there are pronounced inter-species differences in intestinal physiology and molecular signatures that complicate the translation of results obtained in animals to humans (Table 2). While the rat is most readily available animal model of these, it is the species with the largest differences with respect to expression patterns and specificities of drug metabolizing enzymes and drug transporters,[8–10] as well as intestinal physiological parameters compared to humans.[11,12] However, also dogs differ considerably from humans in physiological parameters, such as intestinal pH and the length of villi.[13] In contrast, monkeys are generally considered to more accurately reflect human intestinal anatomy and physiology. Consequently, oral absorption predictions for 56 non-peptide drugs based on rat data were found to be substantially worse compared to results from dogs and monkeys.[14] However, also dogs and monkeys predicted only 54% and 73% of human oral exposure within a four-fold margin.[14] Notably, other studies reported accurate predictions of human oral bioavailability in rats[15] and monkeys.[16]

In contrast to animal models, *in vitro* systems have substantial throughput and cost advantages allowing the automated parallelized screening of tens of thousands of molecules.[17,18] Furthermore, these systems allow to utilize human cells, which eliminates species-specific differences in the molecular machinery governing drug transport and metabolism. In the past decades much research has focused on the development and characterization of methodologically diverse intestinal *in vitro* systems. In this work we give an overview of currently used intestinal cell models (section Intestinal Cell Models for Drug Absorption Studies) and culture paradigms (section Culture Paradigms) and discuss their advantages and limitations with regards to their utility for drug absorption studies. In addition, current developments in the modeling of intestinal pathologies and the extension of intestinal models by integrating the microbiome, mucus or peristalsis are highlighted (section Emerging Trends and Frontiers). Finally, we provide an outlook how these emerging research trends might pave new avenues for studies of drug absorption (section Conclusions And Future Perspectives).

Intestinal Cell Models for Drug Absorption Studies

The intestinal epithelium consists of multiple cell types that are arranged in a columnar layer. Enterocytes are the absorptive cells of the intestinal epithelium and facilitate the controlled uptake of a plethora of molecules from the intestinal lumen, including water, various ions, nutrients and drugs. They constitute the majority of epithelial cells and can be easily recognized morphologically by their microvilli that form brush borders on the mucosal side of the enterocyte.

The most widely used enterocytic cell model are Caco-2 cells, which are derived from human epithelial colorectal adenocarcinoma cells. Upon long-term culture in confluent monolayers, Caco-2 cells differentiate into intestinal enterocyte-like cells with mature features, such as polarization, apical brush borders and formation of tight junctions.[19] Notably, expression of a variety of important ABC drug transporters, including MDR1 and MRP1-6 correlated very well ($r^2 = 0.9$) between Caco-2 cells and jejunal biopsies, whereas expression of BCRP was 100-fold lower in culture.[20] Similarly, expression of most SLC transporters, such as OCT1, OCTN2 and MCT1, was similar between intestinal biopsies and Caco-2 samples, while OATPB levels were >10-fold higher in Caco-2 cells.[21] Drug metabolizing enzymes, such as sulfotransferases (SULTs), UDP- glucuronosyltransferases (UGTs) and cytochrome P450s (CYPs) were expressed at levels pivoting around those found in enterocytes *in vivo*.[22–24] Importantly, Caco-2 culture is highly sensitive to variations in culture conditions, supplements and source of clones and those differences can have pronounced effects on gene expression and functional readouts, which can impair result comparability between different laboratories.[25,26]

Recently, intestinal epithelial cell lines created from biopsies of healthy and diseased individuals have been presented.[27] These lines could be readily expanded in uniform growth medium containing Wnt3a, R-spondin and Noggin, and maintained their origin-specific expression patterns upon differentiation. While the authors did not conduct absorption studies, the formation of polarized monolayers and the presence of a secreted mucus layer on transwell membranes raise hopes that these cells might provide an interesting tool to study inter-individual and region specific differences in drug permeability.

In addition to the aforementioned cell models of intestinal origin, a variety of other *in vitro* models have been presented to predict oral drug absorption. Madin–Darby canine kidney (MDCK) and Lewis lung carcinoma-porcine kidney 1 (LLC-PK1) are both derived from kidney epithelium. Both these cell lines form monolayers with intermittent tight junctions, which are therefore qualitatively suitable to study drug translocation across a cellular barrier. However, as these cell lines differ in transporter expression signatures from Caco-2 cells, which, as described above, show good correlations with human intestinal biopsies, these non-intestinal cell models are mostly considered for the evaluation of passive absorption mechanisms.[28–30]

Besides enterocytes, the intestinal epithelium comprises Paneth cells, which play important roles for intestinal immunity, enteroendocrine L-cells, which secrete gastrointestinal hormones, such as GLP1, mucus producing Goblet cells, as well as M-cells found in the Peyer's patches of the small intestine, which deliver antigens to the underlying immune cells in the lamina propria. For drug

Table 1
Features of Transport Routes Across the Intestinal Epithelium.

Feature	Transport Route			
	Passive Transcellular	Active Transcellular	Paracellular	Transcytosis
Type	Diffusion	Carrier-mediated	Diffusion	Receptor-mediated endocytosis
Direction	Bidirectional	Substrate-dependent	Bidirectional	Mucosal-to-serosal
Driving force	Concentration gradient	Active, secondary active co-transport or facilitative transport	Concentration gradient	Active
Capacity	Very high	Substrate-dependent	Low	Very low
Saturable	No	Yes	Yes	Yes
Stereochemistry-dependent	No	Yes	No	Likely
Inhibitable	No	Yes	No	Yes
Cell model-dependency	Cell model independent	Cell model dependent	Cell model dependent	Cell model dependent
Typical molecule features	Lipophilic, lowly ionized	Hydrophilic	Hydrophilic	Macromolecules

Table 2
Overview of Physiological and Molecular Inter-Species Differences That Can Affect Intestinal Drug Permeability.

	Human	Dog	Rat
Gastric pH	1.7–5.0	1.3–2.1	3.2–3.9
Intestinal pH	6.0	7.3	<6.6
Villi length	Reference	Longer than in human	Similar to human
Bile salt concentration (cholic acid equivalents)	2–5 mg/mL	Higher than in human	10 mg/mL
Thickness of the unstirred water layer (μm)	83–188 μm	35–50 μm	300–500 μm
β-glucuronidase activity (per g of intestinal content)	0.02–0.9	N/A	304–1341
Intestinal CYP3A (pmol/mg protein)	20–30	15	3–4
Intestinal MDR1 (relative expression)	1	Similar to human	0.03
Intestinal MRP3 (relative expression)	1	N/A	0.1
Intestinal UGTs (relative expression)	1	0.1	0.08
Intestinal NTs (relative expression)	1	N/A	0.03

UGTs = UDP-glucuronosyltransferases; NTs = nucleoside transporters; N/A = not available.
Data obtained from Refs.[7,9,11,147–153]

absorption studies, particularly the latter two cell types can be of relevance. The mucus secreted by Goblet cells can act as an additional barrier that modulates drug absorption, particularly of peptide drugs and nanoparticles,[31,32] while M-cells are major sites of transcytosis and can thus be important for absorption studies of macromolecules.[33] Goblet-like cells can be modeled *in vitro* using HT29-MTX cells, which feature apically clustered mucin granules as well as a layer of Alcian Blue positive acidic mucus with an approximate thickness of 20 μm,[34] which is however substantially thinner than the thickness of the human colonic mucus layer (600 μm). Similarly, it has been shown that co-culture of Caco-2 cells and murine B lymphocytes from Peyer's patches (Raji B cells) can induce an M-cell-like phenotype with downregulated AP1 and accentuated transport of micro- and nanoparticles.[35–37]

In addition to the choice of cells, the selection of culture medium and its supplements can have important impacts on intestinal permeability. Particularly, the addition of albumin or serum can have strong influences on the free unbound fraction, which in turn can influence the permeability of the drug in question. For instance, albumin increased apical-to-basolateral and decreased basolateral-to-apical transport a direct function of protein binding of the molecule in question.[38] Furthermore, the authors show that active secretion of compounds with high protein binding might be overestimated in conventional Caco-2 cultures without BSA. Notably, while lipophilicity is often considered to be the most important determinant of plasma protein binding, we want to emphasize that its correlation across chemically diverse compounds is poor, due to a variety of other physicochemical factors, such as polar surface area or pKa, that affect protein binding.[39,40] Similarly, addition of bile acids or other additives that affect drug solubility or free fraction have to be taken into consideration when interpreting the results of permeability studies and, especially, for the quantitative comparison of results across studies.

Culture Paradigms

Static Transwell Cultures

To emulate intestinal drug absorption, transwell culture, in which the cell model of choice is cultured on a permeable filter support, constitute the most established paradigm (Fig. 1A). Developed more than 30 years ago, this model has been extensively used to study intestinal permeability.[41–43] Over the years, this culture paradigm has been subject of a multitude of excellent and comprehensive reviews (for example[44,45]) and detailed protocols are provided that facilitate assay setup and allow for reliable quantification of permeability coefficients.[46] In addition, recent comprehensive proteomics characterizations have increased our phenotypic understanding of the Caco-2 transwell culture model and provide a quantitative overview of their molecular phenotype.[47] While these studies reveal clear differences between Caco-2 cultures and human colon and jejunum proteomes, Caco-2 cells expressed virtually all expected enterocytic markers, as well as 132 different transporters (Fig. 1B).

Most relevantly for the scope of this review is that the model has been carefully characterized for the quantification of oral absorption. Important seminal work was performed by Artursson and Karlsson, who were the first to correlate permeability coefficients of structurally diverse compounds in Caco-2 monolayers grown on permeable filters with oral drug absorption.[48] Using an array of 20 drugs, they found a clear sigmoidal correlation between the experimentally determined apparent permeability coefficients and the absorbed drug fraction in humans *in vivo* that allowed to accurately classify drugs into completely absorbed and poorly absorbed compounds. Moreover, permeability coefficients of Caco-2 transwell cultures correlated well (Pearson r = 0.94) with results from *in situ* perfused rat ileum for a set of 7 structurally dissimilar peptides, corroborating that Caco-2 monolayers constitute a suitable model for the analysis of passive transcellular transport across the intestinal barrier.[49,50]

However, while highly predictive for passive transcellular transport, the prediction of the bioavailability of drugs transported via the active transcellular or paracellular pathway was drastically underestimated.[51] The underlying reasons for these differences are the reduced expression of certain transporters, such as BCRP or LAT1,[20,52] as well as the decreased paracellular permeability of Caco-2 monolayers that is around 100-fold lower compared to human intestinal segments *in vivo*.[53] As the size of the aqueous pores in the small intestine is larger than in the colon, the latter can be explained by the colonic origin or Caco-2 cells.[54] Consequently, a meta-analysis of published permeability data from 98 compounds with diverse transport pathways showed that Caco-2 *in vitro* data could only predict around 59% of the variance in the absorbed fraction in humans (Fig. 2A; Table 3). In contrast, individual studies with smaller drug panels consistently reported substantially better sigmoidal correlations (see e.g.[55–57] and Fig. 2B). These data thus suggest that inter-laboratory variability is substantial, likely at least in part due to insufficient protocol standardization, which impairs quantitative result comparisons across sites.

In an attempt to mimic the cellular complexity of the intestinal epithelium, the conventional Caco-2 filter model was extended by introducing HT29 cells to mimic effects of mucus-secreting Goblet cells and Raji B cells to induce Caco-2 cells to acquire an absorptive phenotype resembling M cells. Permeability of monolayers of HT29-MTX and Caco-2 monocultures did not differ for most compounds[57] and correlated well with the fraction of drug absorbed in humans (Fig. 2C). However, triple co-culture resulted in reduced transepithelial electrical resistance (TEER) and >10-fold increased absorption of hydrophilic marker compounds (Lucifer yellow) and

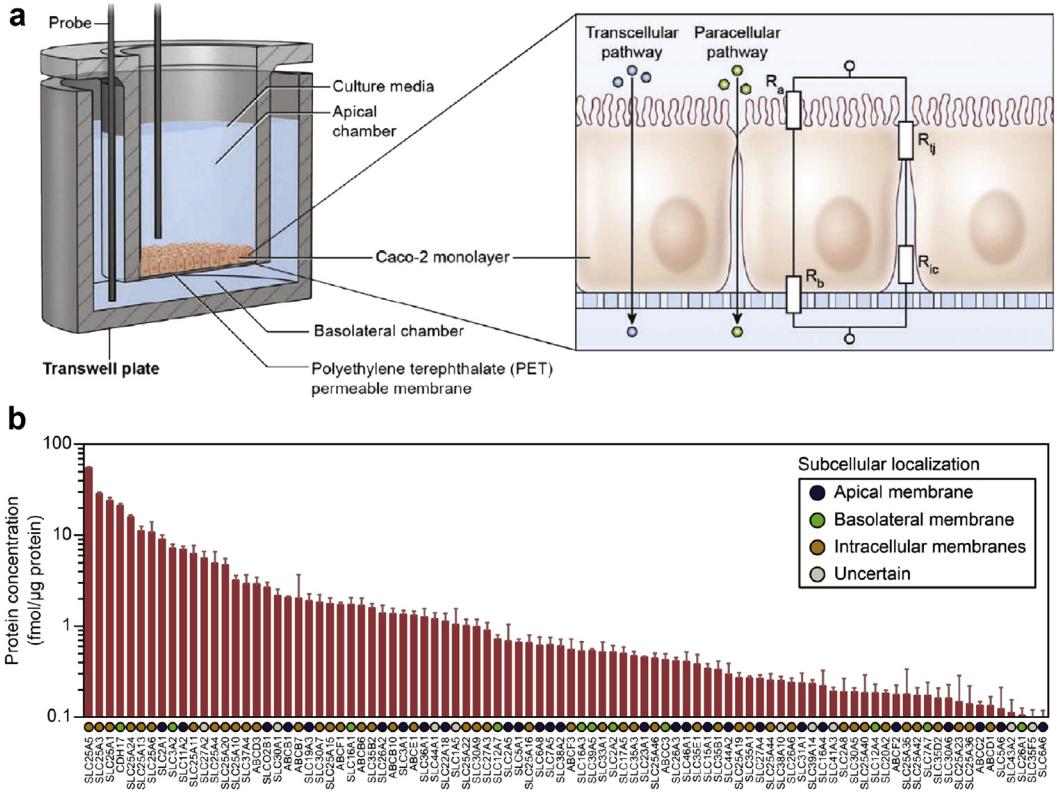

Fig. 1. Caco-2 cells cultured in static transwell cultures express relevant intestinal transporters. A, Schematic diagram of the static transwell culture setup. Intestinal cells are cultured as a monolayer on a permeable filter membrane that separates the upper apical and the lower basolateral compartments. This setup emulates transport via both transcellular and paracellular pathways and allows for facile measurements of transepithelial electrical resistance (TEER). Figure used with permission from Ref.[157] B, Protein concentrations and subcellular localizations of transporters that were present at concentrations >0.1 fmol/mg protein in static Caco-2 transwell cultures after 21 days of culture. Figure modified with permission from Refs.[47]

nanoparticles compared to Caco-2 monocultures.[58–61] Particularly for studies of the latter, triple co-culture systems offer advantages as mucus affects nanoparticle mobility and M cells are epicenters of particulate uptake. Consequently, uptake of nanoparticles in this co-culture model was found to closely correlate with *ex vivo* uptake of porcine intestinal mucosa.[62]

Bioprinting offers interesting possibilities to increase the physiological relevance of transwell cultures. Printing of human primary intestinal epithelial cells and myofibroblasts onto a conventional transwell filter allowed to reconstruct the architecture of the native intestine.[63] Notably, the system exhibited drastically improved expression of drug metabolizing enzymes and transporters and showed adequate TEER within the physiological range. Furthermore, the model accurately classified mitoxantrone, topotecan and propranolol as compounds with low, intermediate and high permeability, respectively.

In summary, static transwell culture systems can be established from multiple cell types either in mono- or co-culture and exhibit similar permeability patterns. *In vitro* permeability measurements of transwell cultures correlate overall almost as well with the absorbed drug fraction in humans as *in vivo* data using rats, dogs or monkeys (compare Fig. 2A–C with Fig. 2D–F). However, drastic quantitative differences exist between *in vitro* models in different laboratories, which render the comparison of absolute absorption values across studies difficult.

Perfusion Systems

While the transwell filter cultures discussed above enable the study of molecular transport across the transepithelial barrier, these systems are static and thus lack intraluminal flow that is characteristic of the intestinal microenvironment *in vivo*. To recapitulate flow, a variety of systems have been developed that perfuse media past intestinal culture models in bioreactors or microfluidic chips.

Already in 2008, Kimura and colleagues presented a microfluidic device made from PDMS with embedded micropumps and an optical fiber insert that allows for continuous pharmacokinetic measurements.[64] In this model, a polyester semipermeable membrane on which Caco-2 cells are cultured, separates apical and basolateral compartments and the authors provide proof-of-concept for polarized transport using rhodamine-123. Imura and colleagues used a similar PDMS microchip in which flow was regulated by microsyringe pumps (Fig. 3A). They analyzed permeability of cyclophosphamide (high permeability) and Lucifer yellow (low permeability) and the apparent permability coefficients of both drugs were not found to be significantly different compared with conventional transwell culture.[65]

Caco-2 cells in co-culture with primary human microvascular endothelial cells exhibit improved morphology, elevated dipeptidase activity and an increase in paracellular transport in perfused compared to static conditions, as evidenced by increased fluoresceine and desmopressine permeability.[66] Quantitatively, transport of caffeine and atenolol, as model drugs for transcellular and paracellular transport, respectively, was substantially higher in perfused culture compared to humans *in vivo*, whereas static transwell culture underpredicted permeability.[67] While the authors argue that perfused models might better mimic the intestinal epithelium in comparison to static transwell models, more extensive studies using a wider range of compounds are necessary to support this hypothesis.

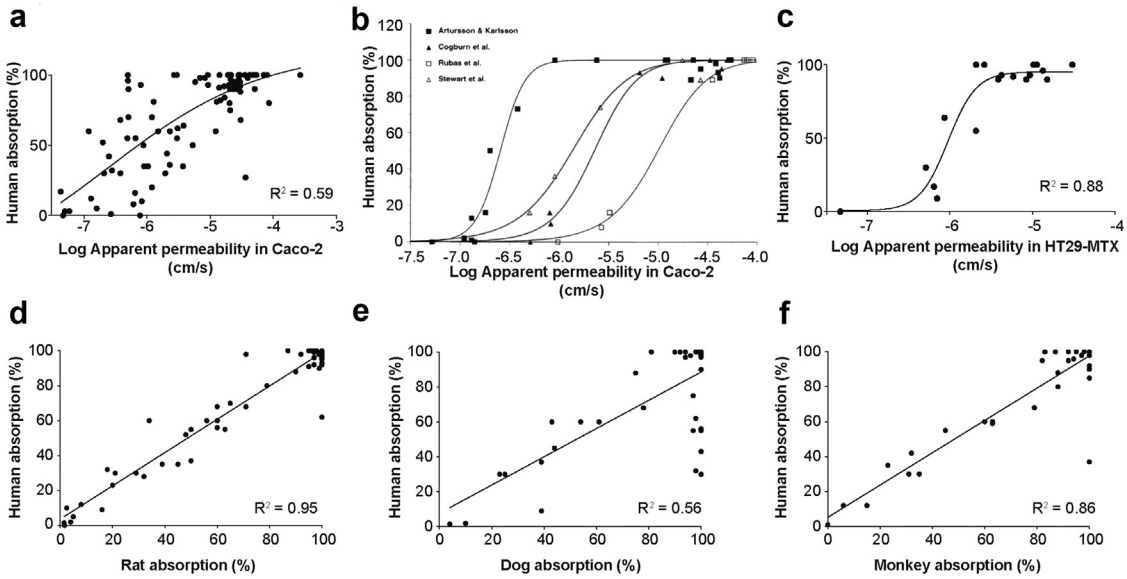

Fig. 2. Static transwell cultures are reliable predictors of intestinal drug absorption. A, Scatter plot depicting the correlation of the apparent permeability of 98 compounds in Caco-2 cell transwell cultures with the fraction of drug absorption in humans. Note that while pooled study results only explain 59% of the variability in human absorption, correlations are substantially improved when individual studies are considered (B; figure used with permission from Refs.[45]). C, The choice of intestinal cell line model has only minor influences on the predictive power of human drug absorption, as evidence by a good correlation of the apparent permeability of transwell monoculture of the goblet-like cell line HT29-MTX with the corresponding absorbed fraction in humans. Notably, the fraction of variability in human drug absorption that can be explained by static transwell cultures is similar to those in commonly used *in vivo* models of rat (D), dog (E) or monkey (F). See Table 3 for individual data points shown in panels A and C–F.

Notably, perfused microchips offer the possibility for highly sensitive quasi-real-time evaluation of drug permeability. A chip consisting of a porous polycarbonate membrane covered with Caco-2 cells and sandwiched between two PDMS layers was coupled to an electrospray ionization quadrupole time-of-flight mass spectrometer for rapid evaluations of drug permeation.[68] Using curcumin as a model drug, the setup correctly classified its low intestinal permeability within 30 min using as little as 6 μl of analytical volume. Drug absorption was furthermore studied using the μCCA model made from silicon dioxide and polycarbonate, which support medium recirculation, and recapitulates human *in vivo* residence time, fluid to tissue ratios, and cellular shear stress.[69,70] Specifically, the authors demonstrate rapid trans-epithelial transport and intestinal metabolism of acetaminophen and suggest that the system might be useful to mimic the pharmacokinetics of orally administered medicines.

A different conceptual modification is the incorporation of peristalsis. Kim and colleagues cultured enterocytic cell models on a stretchable PDMS membrane that separates two microchannels, surrounded by vacuum chambers.[71] Rhythmic application of suction pressure in the vacuum chambers results in mechanical deformations of the monolayer and increased cell strain resembling effects caused by peristaltic motions in the human intestine *in vivo* (Fig. 3B). Strikingly, the combination of peristalsis and liquid flow resulted in the formation of villi-like structures within the monolayer and was sufficient to cause differentiation of enterocytic Caco-2 cells into other intestinal epithelial cell subtypes, including Goblet, enteroendocrine and Paneth cells, at approximately physiological stoichiometries.[72] In addition to Caco-2 cells, the model has been shown to support long-term culture of epithelial cells isolated from dissociated iPS-derived intestinal organoids[73] and primary human epithelial cells.[74] Flow increased TEER compared to static transwell cultures by 3-fold, while peristaltic motions resulted in 4-fold higher paracellular permeability, as measured by fluorescent dextran transport (Fig. 3C and D). Furthermore, villi-like structures exhibited CYP3A4 expression and activity orders of magnitude higher than in static transwell culture.[72]

Notably, differentiation of filter-cultured cells into intestine specific cell types was also achieved by using natural intestinal extracellular matrix (ECM) scaffolds under flow conditions.[75] The authors seeded cells isolated from intestinal crypts from healthy human small intestine on transwell filters coated with porcine small intestinal scaffold (Fig. 3E). Application of perfusion in this setup was sufficient to cause differentiation of subsets of cells into Goblet and enteroendocrine cells. Furthermore, similar to the peristalsis model described above, CYP3A4 and MDR1 function was significantly increased (Fig. 3F and G). Functionally, transport of fluorescein and propranolol were decreased, whereas rhodamine-123 transport was increased. It can be assumed that these effects result in an increased predictive power of the perfused model to predict oral bioavailability, particularly for drugs that are transported via the paracellular route or that undergo significant intestinal metabolism. However, further validation studies using a larger panel of reference compounds are needed to support this hypothesis.

Conceptually, the abovementioned microfluidic intestinal systems can be seen as an extensions of the classical transwell culture in that they incorporate perfusion to the apical and/or basolateral side of the filter culture to mimic intraluminal flow. By contrast, other models aim at recreating the architecture and geometry of the human intestinal epithelium by using hydrogel scaffolds from agarose, collagen, polyethylene glycol diacrylate or poly-ethylene-*co*-vinyl-acetate onto which Caco-2 cells alone or in co-culture with HT29 cells were seeded and cultured for multiple weeks.[76–79] These models can recapitulate the shape, size and density of human intestinal villi, and the available data indicates increased TEER, positive impacts on CYP3A4 expression and transporter function, as evidenced by active glucose transport and increased polarization of rhodamine-123 translocation. A recently developed rotation based setup in which flow direction across the intestinal epithelium alternates between apical-to-basolateral and basolateral-to-apical

Table 3
Comparative Overview of Apparent Permeability in Human Static Transwell Models and Bioavailability in Animal Models and Humans.

Compound	P_{app} * 10^6 in Caco-2 Cells (cm/sec)	P_{app} * 10^6 in HT29-MTX Cells (cm/sec)	Bio-Availability (Human; %)	Bio-Availability (Rat; %)	Bio-Availability (Dog; %)	Bio-Availability (Monkey; %)
Acarbose			1.5	1.5	4	
Acebutolol	0.51		90			
Acetaminophen	9.22		100	98	94	
Acetylsalicylic acid	30.67		68			
Acyclovir	0.38		30	21	100	
Adefovir			12	8		
Alfentanil	270		100			
Alprazolam	23.4		90			
Alprenolol	25.3		94			
Amoxicillin	0.8		93			
Amphotericin B			5	5		
Antipyrine	28.2		100	100		
Atenolol	0.2		52	48		45
Atropine sulfate	8.27		98			
Azipranone			100			95
Azithromycin	1.04		35	45		
Benazepril			37	50	39	32
Benzylpenicillins	1.96	0.51	30			
Bepridil			99			83
Bisoprolol			100	96	98	100
Bretylium			23	20		
Bromocriptine			28	32		35
Caffeine	50.5	30.5	100	100		100
Camazepam			99	97	100	
Captopril			68	71		79
Carbamazepine	17.01		84			100
Carfecillin			100	95		
Cefadroxil			100	95		
Ceftriaxone			0			0
Cephalexin	0.5		96			
Chloramphenicol	20.6	3.86	90			
Chlorpromazine	5.3		50			
Chlorthiazide			56	60	100	
Cimetidine	3.06	0.86	62	100	98	
Cisapride	19.2		100	100		
Clodronic acid	0.059		3			
Clofibrate			96	100		
Clonidine	30.1	13.4	95	100		
Codeine			95	100		
Colchicine	2.08		44			
Corticosterone	54.5		100			
Coumarin			100		81	87
COUNT	98	17	180	79	43	44
Creatinine	1.2		70			
Cyclobenzaprine			100			100
Cyclosporine	0.9		35	39		
Desipramine	21.6		100			
Dexamethasone	23.4	4.22	92			
Diazepam	70.97		100			
Diclofenac			100	100		
Didanosine	0.25		42			
Digoxin	1.28		81			
Diltiazem	29.8		92			
Doxazosin			100	100	81	
Doxorubicin	0.16		5			
Droloxifene			100			100
Enalapril	2.31		60	34	61	
Erythromycin	3.73		35			
Ethinyl Estradiol			100	100	100	
Etiodronate			12			6
Etoposide	1.5		60			
Famotidine			45		44	
Felodipine	9.2		100	100		
Fenclofenac			100	100		
Fluconazole	29.8		100			
Flumazenil			95	100		
Flunisolide			100			100
Fluoxetine	86		80			
Fluvastatin			98	100	100	100
Foscarnet	0.043		17			
Fosinopril			30		25	
Furosemide	0.12		60	60	54	60

(*continued on next page*)

Table 3 (*continued*)

Compound	P_{app} * 10^6 in Caco-2 Cells (cm/sec)	P_{app} * 10^6 in HT29-MTX Cells (cm/sec)	Bio-Availability (Human; %)	Bio-Availability (Rat; %)	Bio-Availability (Dog; %)	Bio-Availability (Monkey; %)
Gabapentin			80	79		
Gancyclovir	0.61	0.7	8			
Glycine	80		100			
Glycylsarcosine	0.5		100			
Granisetron			100	100	100	
Griseofulvin	36.6		27			
Guanabenz	20.9		75			88
Hydrochlorothiazide	0.51		70	65		
Hydrocortisone	14	5.87	91	95		
Ibuprofen	52.5		100			
Imipramine	14.1		100	100		
Indomethacin	20.4		100			
Iothalamate			1.9	4	10	
Irbesartan			100			92
Isoxepac			98	99	100	
Isradipine	19.7		92	100		
Ketanserin			100	100	100	
Ketoprofen			100	100		
Ketorolac			100	87		
Labetalol	9.31		93			
Lactulose	0.27		1			
Latanoprost			100			100
Levodopa			100	97.2		
Lisuride			100			100
Lobucavir	0.88		50			
Lorazepam	20		93			
Lormetazepam			100			100
Lovastatin			30	29	23	31
Mannitol	0.65	0.64	16			
Menogaril			59			63
Menogaril			59			63
Metformin	0.66		55			
Methotrexate	1.2		20			
Methyldopa			43		100	
Methylprednisolone	14.6		82			
Metolazone	3.8		64			
Metoprolol	23.7		95			92
Miglitol			100	100	100	
Morphine			100	100		
Moxestrol			100			100
Moxifloxacin			95			82
Nadolol	0.28		32	18	98	23
Naloxone	14.1	10.3	100			
Naltrexone			100			100
Naproxen	39.5		98	92		
Nicotine	19.4		100			
Nimodipine			100	100	100	
Nisoldipine			97			97
Nitrendipine			88	90	75	
Nizatidine			100	100	99	
Noloxone	28.2		91			
Olanzapine			75		97	
Olsalazine	0.05		3			
Omeprazole	28.5		97	100	100	
Orphenadrine	5.8		95			
Ovastatin			30	29	23	31
Oxatomide			99	100		
PEG4000	0.78		0	1.7		
PEG900	0.83		10	2.5		
Pelrinone			98	71	96	
Phenglutamide			100	100		
Phenobarbital	23.58		90			
Phenylalanine	2.68	2.08	100			
Phenytoin	26.7	15.1	90			
Pindolol	16.7		92			100
Pirmenol			100			98
Piroxicam	35.6		100			
Prazosin	43.6		100			
Prenalterol			97		94	
Progesterone	23.7	9.3	93	100		
Propranolol	27.5	8.45	90	99	100	100
Quinidine	20.4		80			
Raffinose	0.047		0			
Ramipril			60	56	43	

Table 3 (*continued*)

Compound	P_{app} * 10^6 in Caco-2 Cells (cm/sec)	P_{app} * 10^6 in HT29-MTX Cells (cm/sec)	Bio-Availability (Human; %)	Bio-Availability (Rat; %)	Bio-Availability (Dog; %)	Bio-Availability (Monkey; %)
Ranitidine	0.49		55	63	100	
Recainam			85			88
Remoxipride			100	100	99	
Rifapentine			100			100
Rolipram			100			100
Ropinirole			100			100
Saccharin			97	100		
Salicylic acid	41.9	2.61	100	100		
Sormodren			100	100		
Sulfasalazin	0.13		12			
Sulpiride	0.21		30			
Sultopride			100	100	92	
Sumatriptan	3	2.07	55	50	97	
Tamsulosin			100	100	90	
Tenidap	51.2		90			
Terbutaline	0.38		68	60	78	
Testosterone	72.27	11.33	100			
Theophyline			96	97		
Tiludronate			12			15
Timolol	12.8		81			
Tolmesoxide			100	100	100	
Trovafloxacin	30.23		88			
Valaciclovir	2.3		36			100
Valproic acid	48		100			
Venlafaxine			92	97		
Verapamil	2.98		100	100		
Viloxazine			100	100		100
Warfarin	21.1		97			
Xamoterol			9	16	39	
Ximoprofen			100	100		
Zidovudine	6.93		98			
Ziprasidone	12.3		60			
Zolpidem			100			100
Zomepirac			96			94
Zopiclone			100		100	

Compounds are listed in alphabetic order.
Data collected from Refs.[7,16,26,55,57,154–156]

allows the reconstruction of the typical wrinkle morphology of the intestinal canal.[80] However, the impacts of such approaches on drug absorption have not been assessed systematically.

The majority of devices are comprised of materials, such as PDMS,[81] that significantly absorb drug molecules. As a result, in these devices the concentration of drug molecules in solution can be considerably lower than the input concentration and is subject to change over time. While these effects have to our knowledge not been analyzed for intestinal microfluidic perfusion chips, we refer the interested reader to critical discussions of this topic in the frame of other organ-on-chip models.[82,83] To ameliorate such effects, a membrane-free device has been developed in which a channel lined with Caco-2 cells is separated from an adjacent flow channel by gel made from rat tail collagen.[84] Simple rhythmic tilting of the chip provides a means to induce gravitational flow. While the system has not been used for the study of drug permeability, the authors provide proof-of-concept data for indication of intestinal leakiness using staurosporine and aspirin. Similarly, the use of thiol-ene and Teflon as chip and membrane materials, respectively, promises to ameliorate drug absorption and facilitate rapid device prototyping.[85] The device is compatible with the formation a functional intestinal barrier of Caco-2 cells with permeability values of mannitol, FITC-labeled 4 kDa dextran and insulin, similar to conventional transwell cultures.

In conclusion, a plethora of conceptually diverse fluidic intestinal models have been developed over the last decade. These models have provided impressive advancements regarding the reconstruction of the morphology and cellular complexity of the intestinal epithelium. Furthermore, promising functional data has been presented that indicates that barrier integrity, paracellular permeability and metabolic activity of perfused models might resemble the human intestine *in vivo* more closely than static filter cultures. Importantly however, comprehensive benchmarking of drug transport using a panel of well-characterized reference compounds, as well as systematic benchmarking across model systems is currently lacking.

Organoids

Organoid culture constitutes an emerging culture method that allows to study *ex vivo* analysis of stem cell biology and differentiation. The first intestinal organoids were published by Ootani and colleagues who demonstrated that cultures of intestinal fragments of neonatal mice resulted in the formation of sphere-like structures that exhibited Wnt-dependent proliferation and were capable of multilineage differentiation.[86] In contrast, organoids with a central lumen surrounded by multiple extrusions can be established from primary Lgr5+ stem cells cultured in Matrigel.[87] These structures self-organize into villus-like and crypt-like domains with appropriate cellular compositions of enterocytes, Goblet cells, Paneth cells, and enteroendocrine cells, and are compatible with dissociation and passaging similar to immortalized cell lines (Fig. 4A). However, previous comparative studies showed that transcriptomes of intestinal microfluidic chip cultures were more similar to *in vivo* small intestine compared to organoids (ref.[74] and Fig. 4B).

Intestinal organoids have been used with great success for a range of applications, including studies of intestinal development, stem cell biology, disease modeling and regenerative medicine. As

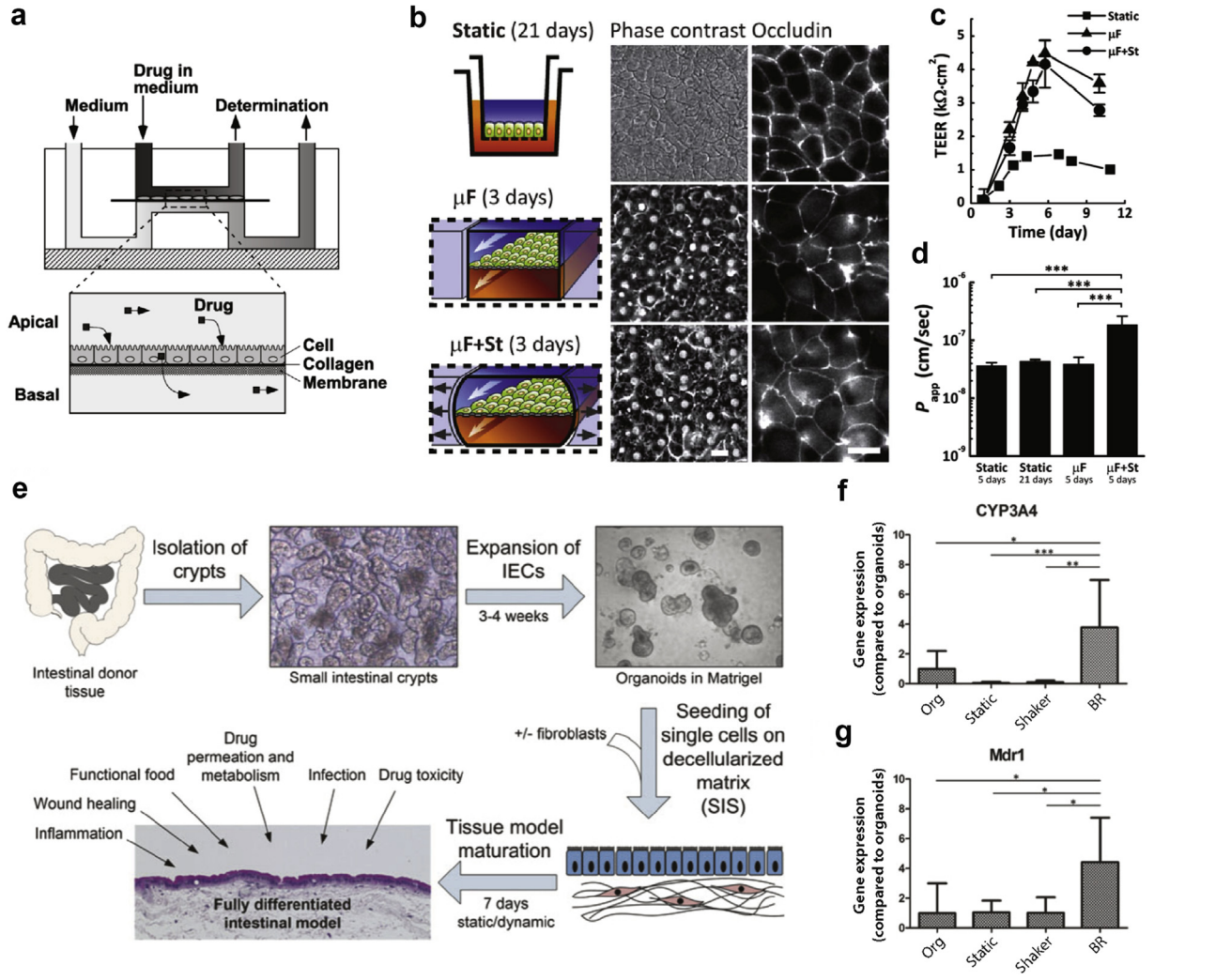

Fig. 3. Microfluidic perfusion facilitates maturation of intestinal epithelial cells. A, Schematic cross-sectional view of a perfusion device for intestinal absorption measurements. In short, two separately perfused channels are separated by a collagen coated filter membrane onto which Caco-2 cells are cultured. Compounds are added to the apical channel and its absorption into the basal compartment can inform about drug permeability. Figure modified with permission from.[65] B, Comparison of static Caco-2 transwell chip with microfluidic chip culture with perfusion (μF) or perfusion + rhythmic stretching mimicking peristalsis (St). Left: schematic depictions of the respective *in vitro* system. Middle: phase contrast images of the monolayer. Right: fluorescence image of the tight junction protein occludin. C, Barrier function of the epithelium, as quantified by TEER, is stimulated by flow whereas mechanical strain does not further increase tight junctional integrity. D, By contrast, the apparent paracellular permeability (P_{app}) of fluorescent dextran of the Caco-2 monolayer depends on mechanical strain and not on flow. *** indicates $p < 0.05$. Panels B–D modified with permission from.[71] E, Schematic depiction of the workflow to generate a small intestine model from primary human crypt organoids. Culture of these organoid (Org)-derived intestinal models on decellularized biological scaffold in perfused bioreactors (BR) increases expression of important drug metabolizing enzymes (F, CYP3A4) and drug transporters (G, MDR1) compared static culture or culture on a shaker, corroborating the importance of physiologically relevant perfusion. Panels E–G modified with permission from.[75]

these applications are not the scope of this review, we refer the interested reviewer to recent comprehensive expert reviews on these subjects.[88–91] Only few intestinal toxicity studies using organoid models have been presented. Using organoids from UGT1A1-deficient and control mice, UGT1A1-dependent glucuronidation was shown to be a key step in the detoxification of the topoisomerase inhibitor SN-38.[92] Importantly however, while they have long been suggested to revolutionize oral bioavailability studies,[93] no prediction data for drug absorption in intestinal organoids has to our knowledge been presented to date, particularly due to technical difficulties arising from their morphology and orientation with a central intestinal lumen that complicates physiologically relevant drug transport studies. As such, the only studies using organoid derived intestinal cells for permeability assessments dissociate the organoids, sort for EPCAM positive cells and seed them on permeable membranes for culture in static transwells[94] of microfluidic devices[73] where they form confluent monolayers with relevant TEER and paracellular permeability (Fig. 4C–E).

Ussing Chamber Systems

The Ussing chamber provides an experimental setup that allows to simultaneously evaluate membrane permeability and transporter activity. The chamber constitutes a device in which an intestinal preparation is mounted vertically to separate two chamber compartments, each of which is superfused using separate buffers, typically on the basis of bicarbonate buffered Ringer solution (Fig. 5A–C). TEER measurements can inform about epithelial permeability and have been successfully used to demonstrate differences in intestinal integrity between normal and inflammatory conditions.[95]

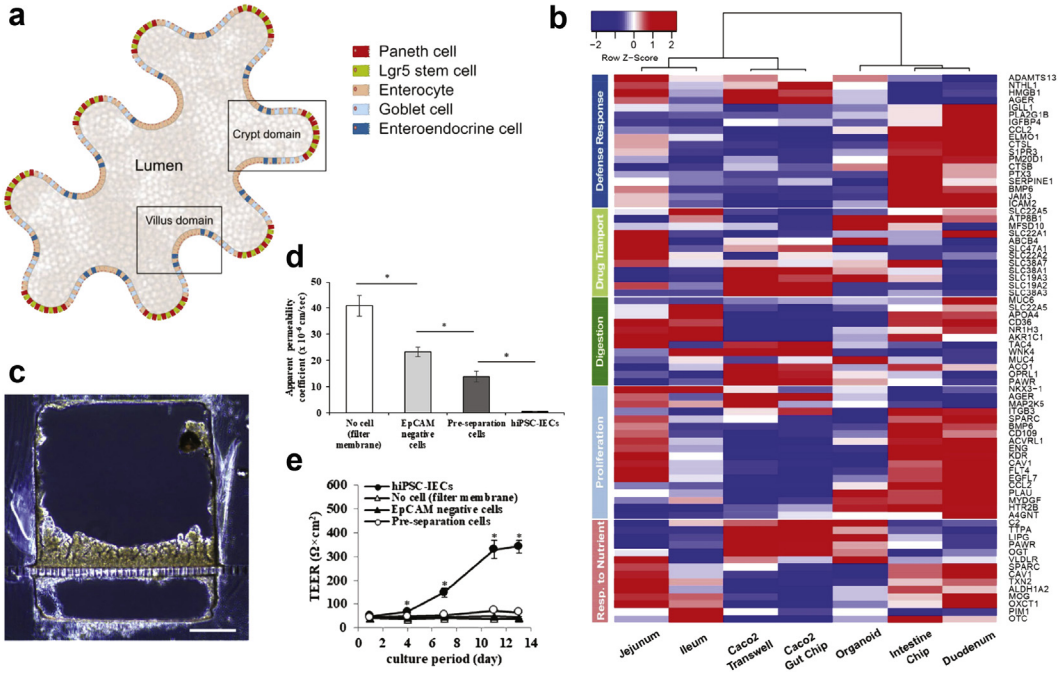

Fig. 4. Organoid culture can recapitulate the cellular complexity of the intestinal epithelium. A, Schematic depiction of an intestinal organoid showing alternating villus and crypt domains surrounding a central lumen. Crypts are enriched in Lgr5+ stem cells and Paneth cells while enterocytes, enteroendocrine, and Goblet cells line are primarily found in the villi. Figure modified with permission from.[158] B, Mean-centered sigma-normalized heatmap representation of transcriptomic differences of various *in vitro* models, as well as human jejunum, ileum and duodenum. Note that 3D organoids and the microfluidic chip refer to the same duodenal tissue biopsies derived from 3 healthy donors. Figure modified with permission from.[74] C, Brightfield image of the cross-section of an intestinal microchip established from human induced pluripotent stem cell (hiPSC)-derived intestinal organoids after 14 days reveals a physiologically relevant architecture with villous-like structures. Scale bar = 250 μm. Figure modified with permission from .[73] Static transwell culture of EPCAM + cells isolated from hiPSC- derived intestinal organoids (hiPSC-IECs) exhibits low apical to basal paracellular permeability of the hydrophilic marker Lucifer Yellow (D) and high levels of TEER compared to EPCAM-cells (E). Data indicate average±S.D. * corresponds to $p < 0.01$ versus the pre-separation cells. Figure modified with permission from.[94]

Using human intestinal and colonic tissue sections, the apparent permeability coefficients of eleven test compounds with different physiochemical properties, correlated well with the human absorbed fraction ($R^2 = 0.87$; ref.[96]). Tissue accumulation of drugs, intestinal metabolism and apical changes in drug concentration due to precipitation can be incorporated into absorption analyses and have been shown to accurately predict intestinal absorption in humans.[97,98] Furthermore, Ussing chamber experiments using tissues from patients with inflammatory bowel disease revealed increased permeability to nanoparticles, which correlates well with *in vivo* observations showing an accumulation of nanoparticles at acute inflammatory foci.[99]

Tissue sections from duodenum, jejunum, ileum and colon could recapitulate clear regiospecific differences in drug absorption.[100] Notably, while some inter-laboratory variability was observed for Ussing chamber cultures, particularly for lowly permeable compounds, the results from different laboratories could be quantitatively combined, resulting in a significant sigmoidal association (Fig. 5D). This is in marked contrast to results from Caco-2 transwell cultures that were only qualitatively similar between laboratories, as discussed above (section Static Transwell Cultures). Combined, these results demonstrate that the Ussing chamber constitutes a robust method to predict human intestinal permeability that is however hampered by the limited availability of fresh human tissue section.

Slice and Explant Cultures

In addition to the *in vitro* cell culture models discussed above, cultures of organotypic intestinal slices of rat, mice, chicken, or human have emerged as an alternative *ex vivo* model to study intestinal drug metabolism, toxicity and transport.[101] The main advantages of this culture method are the maintenance of physiological architecture and cellular complexity (Fig. 6A–C). Furthermore, slices from different regions of the intestinal tract retain their regiospecific molecular and functional differences, thus allowing to compare permeability along the intestinal axis.[102] Notably however, these cultures are only viable and functionally stable for short periods of time with a maximum of 6–24 h, depending on species.[103,104]

While precision cut intestinal slices (PCIS) are most extensively characterized as model systems for acute intestinal toxicity,[105,106] drug metabolism[107,108] and induction,[103] an increasing body of literature also demonstrates their suitability for absorption studies. PCIS from rat were successfully used to assess the potency of various MDR1 inhibitors, including verapamil, indomethacin and glibenclamide, cyclosporine A, quinidine and ketoconazole.[109,110] While PCIS results differed quantitatively from other *in vitro* models, the rank order of the compounds was retained, suggesting a good overall qualitative agreement. Interestingly, parallel incubation of PCIS at 4 °C and 37 °C provides a tool to distinguish between active and passive drug transport (Fig. 6D–F). Furthermore, using inhibitor studies, the authors could confirm ASBT (*SLC10A2*), as the responsible bile acid transporter.[111]

Furthermore, in proof-of-concept studies, murine and human intestinal slice cultures were used to study the effect of oxygen and microbiota on segmental contractility, epithelial proliferation and mucosal immune functions *ex vivo*.[112,113] Integration of *ex vivo* slices into microfluidic perfusion chips opens new possibilities to increase the functional life span of organotypic slice cultures. Richardson and colleagues presented a microfluidic organotypic device (MOD) that supports the culture of mammalian explants,

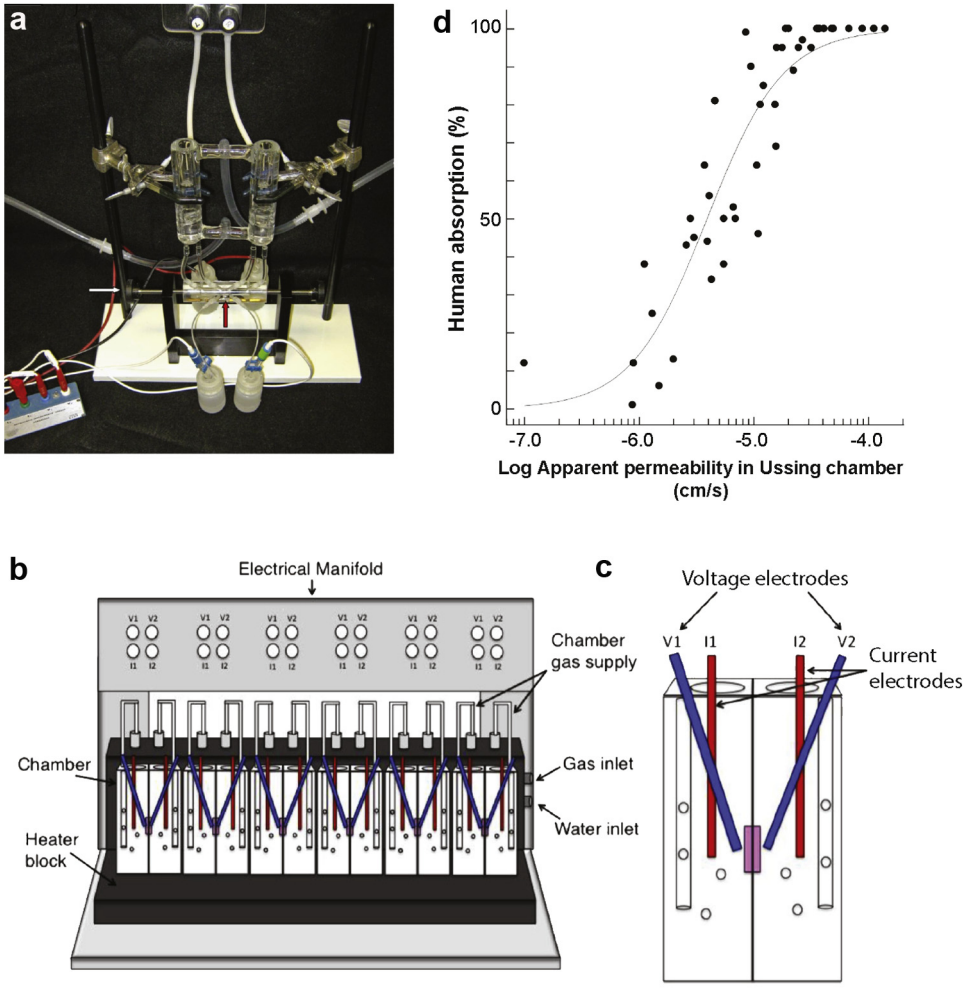

Fig. 5. The Ussing chamber allows robust culture of human intestinal sections with minimal inter-laboratory heterogeneity. A, Overview image of a typical assembled Ussing chamber with water-jacketed reservoirs and a mounted intestinal sample (red arrow) separating the two chamber halves. Figure modified with permission from.[159] B, Schematic of a miniaturized Ussing device with independent chambers, voltage and current control for parallel measurement of up to six intestinal sections. C, Individual Ussing chamber with voltage and current Ag/AgCl electrodes. Electrodes are positioned to be close to each other but not touching within the chamber. Panels B and C modified with permission from.[95] D, Scatter plot depicting the correlation of the apparent permeability measured in Ussing chambers using tissue samples from different donors with the fraction of drug absorption in humans. The results are pooled from three independent studies conducted in different laboratories, highlighting the low inter-experimental variability of the Ussing culture paradigm. Figure modified with permission from.[100]

including muscular, neural, immune, and epithelial components for 72 h[114] up to 8 days.[115] The device consists of three cyclic olefin copolymer (COC) layers that define independent luminal and serosal flow channels that interface at the middle layer onto which the explant is positioned (Fig. 6G). Culture of explants in hypoxic conditions allowed to better support the intestinal microbiota. As such, these systems provide an interesting cross-over between explant culture and microfluidic chip technology and hold promise for their application to drug absorption studies.

Emerging Trends and Frontiers

Intestinal cell models were long used primarily as models for drug permeability across enterocytes and, more recently, more complex and physiologically relevant models of the intestinal epithelium. However, recent emerging trends have significantly expanded this scope. In the following some current trends within the intestinal model space will be presented and it is outlined how these advances could impact drug permeability testing.

Incorporation of Microbiota

The microbiome plays central roles in the absorption and metabolism of nutrients and drugs.[116] Over the past years a large number of studies have cultured intestinal cell models with commensal and pathogenic microbes.[117] However, major challenges for the *in vitro* culture and maintenance of the complex human microbiome, such as the establishment of microbiome homeostasis without elimination or overgrowth and the low oxygen levels required to support microbiome diversity, could only be resolved recently.

Intestinal organoids have been succecssfully used for bacterial monocultures[118–120] and for the culture patient-derived microbial communities.[121] However, these methods require the laborious and technically challenging microinjection of bacteria into the organoid's lumen, which limits throughput or requires major investment into robotic infrastructure. In addition, microinjection setups require medium supplementation with antibiotics to prevent the contamination of culture medium and can only be cultured for short periods of time (<4 d) due to the lack of luminal perfusion and bacterial overgrowth.

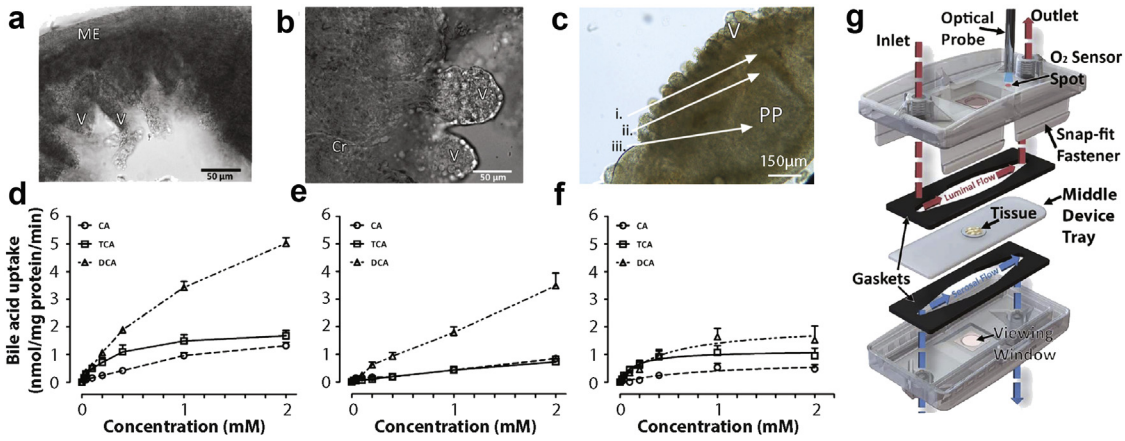

Fig. 6. *Ex vivo* slice cultures maintain the regiospecific intestinal architecture and function. A-B, Organotypic slice culture retains the structural integrity of mouse ileum with clear villi (V) and crypt domains (Cr) and an intact muscularis externa (ME) for at least 48 h in culture. C, Similarly, Peyer's patches (PP) with follicle-associated epithelium (i), subepithelial dome (ii) and the germinal center (iii) remained intact for at least 24 h, opening possibilities to study acute immunological responses *ex vivo*. Panels A–C modified with permission from.[112] D-F, Incubation of precision cut intestinal slices at different temperatures allows to dissect transport mechanisms. Uptake of cholic acid (CA), taurocholic acid (TCA) and deoxycholic acid (DCA) in rat ileum slices at 37 °C (corresponding to the sum of active and passive uptake; D) and 4 °C (corresponding to passive uptake only; E) is dose-dependent. Based on these data, the contribution of active transport can be calculated by subtraction (F). Panels A–C modified with permission from.[111] G, Layer-by-layer model of a microfluidic device to support the culture of *ex vivo* intestinal slices. The system features independent luminal (red) and serosal (blue) flow paths, as well as integrated oxygen sensors. Figure modified with permission from.[114]

Importantly, a recent study using a two-channel microfluidic gut-on-a-chip device with Caco-2 cells under dynamic fluid flow and peristalsis-like mechanical deformations, allowed for the first time to culture and maintain the complexity of the commensal gut microbiome for up to one week.[122] The authors describe the establishment of an oxygen gradient that supports the culture of both aerobic and anaerobic microorganisms for up to one week. Furthermore, overall microbial diversity could be sustained and phyla composition resembled those found in human microbiome stool samples. While the authors did not evaluate drug metabolism or permeability, the experimental setup certainly constitutes a promising tool to parse the effects of inter-individual differences in microbial composition on intestinal barrier function.

Disease-Modeling

An increasing number of inflammatory diseases and microbial pathogens have been associated with reduced intestinal barrier function and the modeling of such gastrointestinal pathologies constitutes a major frontier of current research.[123] Disease models have been presented based on static or perfused transwell cultures as well as organoids, whereas the short life-span of tissue slices prohibits the induction of intestinal pathologies *ex vivo* and is thus limited to tissue samples obtained from already injured intestines.[124] Key advantages of organoid cultures for the study of intestinal diseases are the possibility to expand cells isolated from patient biopsies almost unlimitedly, thus allowing high-throughput screening studies in a phenotypically relevant human context. Intestinal organoids are susceptible to infection with rotavirus, echovirus 11, coxsackie virus B1 and enterovirus 71, whereas they replicate only poorly and do not induce antiviral and inflammatory signaling in intestinal cell lines.[125,126] Organoids also support the complex life cycle of the obligate parasite *Cryptosporidium parvum* when microinjected into the lumen, thus posing a unique paradigm for the study of this clinically highly relevant pathogen.[127] Moreover, organoids are compatible with high-throughput methods. Robotic microinjection allows to study patient-derived microbial community dynamics, as well as their effects on barrier function,[121] while automated imaging and analysis of the swelling of organoids, facilitates the identification of compounds that promote or inhibit the flux of water and ion across the epithelial barrier.[128]

In contrast to organoids, transwell cultures allow easy access to both apical and basolateral compartments. Transwell cultures have been successfully used to study effects of inflammation on villus injury and barrier function. Specifically, lack of peristalsis resulted in bacterial overgrowth, which triggered expression of proinflammatory cytokines and compromised barrier integrity.[129] Similarly, barrier function was reduced in a hypoxic environment upon challenge with IL1β and TNFαa[130,131] Furthermore, transwell cultures constitute pathophysiologically relevant models to study disease mechanisms and molecular consequences of a range of enteric infections, including coxsackie B1 virus,[132] norovirus,[133] human adenovirus[134] and Enterohemorrhagic *Escherichia coli*.[135]

Table 4
Advantages and Limitations of Current Intestinal *In Vitro* Culture Systems.

	Transwell Cultures	Perfusion Systems	Organoids	*Ex Vivo* Cultures
Available drug permeability data	***	*	–	***
Molecular and cellular phenotype	*	**	**	***
Temporal stability	**	**	***	–
Throughput	***	**	***	*
Ease of use	***	*	*	**
Costs	***	*	***	**
Model versatility	**	***	***	*
Utility for absorption studies	***	**	*	***

The abovementioned studies provide a glimpse of the considerable impacts of how intestinal *in vitro* models have already advanced the molecular understanding of enteric disease mechanisms. However, while effects of inflammation and infection on epithelial permeability are plausible, e.g. due to changes in cellular architecture, tight junction alterations, localized necrosis or altered mucus production, only few studies have directly analyzed the effects of these disease models on drug absorption. In a co-culture of Caco-2 cells with human macrophage and dendritic cells on a transwell filter, exposure to lipopolysaccharides from *Escherichia coli* and *Salmonella typhimurium* or the inflammatory cytokine IL1β activated immune cells, increased fluorescein transport across Caco-2 monolayers and sensitized enterocytes to nanoparticle cytotoxicity.[136,137] Furthermore, encapsulation of colistin, an otherwise poorly permeable compound, into liposomes coated with the extracellular adherence protein (Eap) of *Staphylococcus aureus* facilitated intracellular drug delivery and resulted in a significant reduction of bacterial load in *Salmonella enterica* infected Caco-2 cell transwell cultures.[138]

Peristalsis

The periodic stretching and contracting of the intestinal epithelium by peristaltic movements causes repetitive strain that impacts on cellular functions. Already in 1996 Basson and colleagues showed that physiologically relevant rhythms and magnitudes of deformation of a Caco-2 cell membrane culture, stimulated cell proliferation and induced expression of the brush border enzyme dipeptidyl dipeptidase.[139] Furthermore, cyclic stretch disrupted tight junction and adherens junction integrity and increased paracellular permeability in Caco-2 transwell cultures.[140] Notably, the development of a microfluidic model that allows to emulate peristalsis-like cyclic stretching while supporting long-term culture[71,72,74] thus holds promise to improve drug absorption predictions, particularly of hydrophilic compounds that are primarily absorbed via the paracellular pathway. However, comprehensive screening of drug absorption across a diverse set of training compounds in an *in vitro* model undergoing cyclic strain has to our knowledge not yet been reported.

Mucus

Mucus is a complex hydrogel consisting primarily of water and a multitude of glycoproteins of which mucins are most abundant.[141,142] Intestinal mucus is produced by Goblet cells and can alter drug absorption kinetics by interacting with drugs and reducing their diffusion coefficients. Examples of compounds that bind extensively to gastrointestinal mucus are isoniazid, pentamidine, rifampicin, *p*-aminosalicylic acid and pyrazinamide, all of which show an at least 10-fold reduction in apparent permeability when diffusing through a 3 mm mucus layer compared to 3 mm of buffer solution.[143] Overall, the most important predictor of the mucus diffusibility of drugs was lipophilicity, whereas size only had considerable effects for very large molecules.[144,145]

In light of the preamble above, it is not surprising that incorporation of mucus into drug absorption studies has been subject of considerable interest. Transwell co-cultures of Caco-2/HT29 cells reduced the permeability of the lipophilic barbitals and testosterone compared to Caco-2 monocultures despite having significantly reduced levels of TEER.[34] However, other studies showed discrepant results, possibly due to altered mucus thickness or composition due to the use of a different Caco-2 subclone (TC7) or of altered stoichiometries between enterocyte- and goblet cell-like cells.[57] Notably, the mucus layer of static transwell cultures is overall much thinner than in the intestine *in vivo* (<50 μm compared to approx. 600 μm), suggesting model-related differences in drug permeability and absorption.

One interesting recent advancement is the finding that goblet cells spontaneously differentiate in a microfluidic model at physiologically relevant stoichiometries, as judged by expression of SPDEF, KLF4 and various mucus granule proteins.[146] In contrast to previous approaches, this system produces mucus layers of 300 μm, which further swell to 600 μm upon 6-day exposure to prostaglandin E2, a factor implicated in alterations of mucus physiology *in vivo*, due to increased Na−K−Cl cotransporter activity. While mucus thickness resembles the intestinal environment *in vivo*, mucus composition in this model has not yet been analyzed. Taken together these data suggest that currently available models can emulate physiological effects of mucus on drug absorption. However, unlike for static transwell cultures, a direct demonstration of this hypothesis has not been presented.

Conclusions and Future Perspectives

In vitro cell culture systems of the gastrointestinal tract are widely used as preclinical models to predict oral drug absorption. A variety of conceptually distinct culture methods have been developed over the last 30 years (Table 4). Particularly transwell cultures of Caco-2 cells, alone or in co-culture with other cell lines, have been extensively tested and benchmarked. Results of these studies showed that static Caco-2 transwell cultures are good qualitative predictors of the absorbed fraction in human, particularly for hydrophobic drugs that are absorbed via the passive transcellular route. Results from transwell systems using cell lines showed an overall good correlation with absorption data from animal models, are considerably cheaper than animals and offer the possibility for high-throughput screenings, which explains their implementation into the early preclinical testing arsenal. Furthermore, substantial species-specific differences in absorbance can be observed for some compounds, particularly in canine models, rendering them relatively poor predictors of human drug absorption. A variety of physiological factors have been suggested to explain these discrepancies, including differences in absorptive area, transit time and intestinal pH. However, intestinal *in vitro* models using cells derived from preclinically important model species that could be used to study these species differences have not been presented and thus our understanding of the molecular underpinnings of inter-species differences in drug absorption remains limited.

While static Caco-2 transwell cultures are widely used to predict human drug absorption *in vivo*, they do not recapitulate a variety of physiological features of the intestinal tract *in vivo*, including luminal flow, peristaltic movements, the cellular complexity of the intestinal epithelium as well as effects of mucus and microbiota on intestinal drug metabolism and absorption. It is thus intuitive that *in vitro* models that incorporate of these factors might exhibit a further improvement in their predictive performance. Tremendous progress in the development of organotypic and microphysiological intestinal models based on organoids, microfluidic chips, bioreactors or *ex vivo* slice cultures that can mimic these aspects has been made in recent years. Importantly however, while these models have already provided significant insights into intestinal biology and pathobiology, their systematic characterization for drug absorption is lagging behind. It is thus as of yet unclear whether these advanced intestinal systems add sufficient value for permeability predictions compared to conventional static transwell cultures to justify the considerable increase in model complexity.

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