



# Oral absorption of peptides and nanoparticles across the human intestine: Opportunities, limitations and studies in human tissues<sup>☆</sup>



P. Lundquist, P. Artursson<sup>\*</sup>

Department of Pharmacy, Uppsala University, Box 580, SE-752 37 Uppsala, Sweden

## ARTICLE INFO

### Article history:

Received 2 May 2016

Received in revised form 2 July 2016

Accepted 8 July 2016

Available online 3 August 2016

### Keywords:

Oral drug delivery

Peptide drugs

Nanoparticles

Ussing chamber

Peptide permeability

Bioavailability

Human intestinal tissue

Human Protein Atlas

Transcytosis

Transepithelial transport

## ABSTRACT

In this contribution, we review the molecular and physiological barriers to oral delivery of peptides and nanoparticles. We discuss the opportunities and predictivity of various *in vitro* systems with special emphasis on human intestine in Ussing chambers. First, the molecular constraints to peptide absorption are discussed. Then the physiological barriers to peptide delivery are examined. These include the gastric and intestinal environment, the mucus barrier, tight junctions between epithelial cells, the enterocytes of the intestinal epithelium, and the subepithelial tissue. Recent data from human proteome studies are used to provide information about the protein expression profiles of the different physiological barriers to peptide and nanoparticle absorption. Strategies that have been employed to increase peptide absorption across each of the barriers are discussed. Special consideration is given to attempts at utilizing endogenous transcytotic pathways. To reliably translate *in vitro* data on peptide or nanoparticle permeability to the *in vivo* situation in a human subject, the *in vitro* experimental system needs to realistically capture the central aspects of the mentioned barriers. Therefore, characteristics of common *in vitro* cell culture systems are discussed and compared to those of human intestinal tissues. Attempts to use the cell and tissue models for *in vitro*–*in vivo* extrapolation are reviewed.

© 2016 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

## Contents

1.	Introduction	257
2.	Peptide drugs	257
3.	The barriers	257
3.1.	The gastric and intestinal digestive environment	257
3.2.	The mucus barrier	259
3.2.1.	Mucus barrier structure	259
3.2.2.	Mucus interactions	260
3.3.	Tight junctions	260
3.3.1.	Tight junction structure and function	260
3.3.2.	Tight junction modulation for oral peptide delivery	261
3.4.	Intestinal epithelial cells	262
3.4.1.	Cell penetrating peptides	262
3.4.2.	Endocytosis and transcytosis	263

**Abbreviations:** EIPA, 5-(*N*-ethyl-*N*-isopropyl) amiloride; AMN, amnion-less; AMP, adenosine monophosphate; AEE, apical early endosome; ARE, apical recycling endosome; BRE, basal recycling endosome; BFA, Brefeldin A; ClogP, calculated log P; C8, caprylic acid; CPP, cell penetrating peptide; CCK, cholecystokinin; CLDN, claudin; CUBN, cubilin; cAMP, cyclic AMP; CFTR, Cystic fibrosis transmembrane conductance regulator, ABCC7; DPP, dipeptidyl-peptidases; ER, endoplasmic reticulum; Fa, fraction absorbed; Fc, constant domain of Ig; GLP-1, Glucagon-like peptide-1; GLUT2, glucose transporter 2; ENPEP, glutamyl amino peptidase; Ig, Immunoglobulin; IL, interleukin; IBD, inflammatory bowel disease; IU, international unit; IF, intrinsic factor; LDH, lactate dehydrogenase; ITLN-1, Lactoferrin receptor; LE, late endosome; LOQ, limit of quantitation; LY, lysosome; MDCK, Madine-Darby Canine Kidney cells; M-cells, microfold cells; MME, membrane metallo-endopeptidase; MUC, mucin; MPP, mucus penetrating particle; MLC, myosin light chains; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; FcRn, FcγR1, Neonatal Fc receptor; NDA, new drug application; OCLN, occludin; PNLIP, Pancreatic lipase; PBS, phosphate buffered saline solution; PSA, polar surface area; PLGA, poly (D,L-lactic-co-glycolic acid); PIGR, Polymeric immunoglobulin receptor; TMPRSS15, transmembrane protease serine 15; MARVELD2, tricellulin; TAT, trans-activating transcriptional activator; TNF-α, tumor necrosis factor alpha; US FDA, US Food and drug administration; ZO-1, zonula occludens 1.

<sup>☆</sup> This review is part of the *Advanced Drug Delivery Reviews* theme issue on "SI: Oral delivery of peptides".

<sup>\*</sup> Corresponding author at: Department of Pharmacy, Uppsala University, Sweden.

E-mail addresses: [patrik.lundquist@farmaci.uu.se](mailto:patrik.lundquist@farmaci.uu.se) (P. Lundquist), [per.artursson@farmaci.uu.se](mailto:per.artursson@farmaci.uu.se) (P. Artursson).

3.4.3. Targeting to transcytosed receptors . . . . .	265
3.4.4. Nanoparticles and M-cell mediated transcytosis. . . . .	266
3.5. Subepithelial tissue. . . . .	267
4. Caco-2 cells as an in vitro model system of intestinal absorption. . . . .	267
4.1. Novel advanced in vitro model systems . . . . .	268
5. Ussing chambers . . . . .	268
5.1. Translation from Ussing chamber experiments to in vivo intestinal absorption . . . . .	270
6. Modeling of jejunal peptide or nanoparticle absorption. . . . .	271
7. Summary and conclusions. . . . .	272
Acknowledgment . . . . .	272
References . . . . .	272

## 1. Introduction

The main physiological function of the gastrointestinal tract is to digest food constituents that can subsequently be absorbed as nutrients and at the same time provide an efficient barrier to toxic materials, including peptides, viruses and bacteria. Whether administered in soluble form or formulated in nanoparticles, these two functions are clearly at odds with the oral delivery of large molecules such as peptides and proteins. It is, therefore, not surprising that oral delivery of peptide drugs remains a challenge. While, in theory, nanoparticles have a good potential to enable the oral delivery of peptides and proteins, the design of a nanoparticle able to survive passage through the gastrointestinal tract and to pass the intestinal epithelium is in itself a significant challenge [1–3].

In this contribution, we first review current knowledge regarding the molecular properties required for oral absorption of peptides. We then discuss the physiological barriers to oral delivery of peptides and nanoparticles. Examples of principles that have been used to enhance peptide and nanoparticle delivery across each of these barriers are provided. For a comprehensive review of nanoparticle design for oral delivery see [1]. Finally, we also investigate the suitability of human intestinal tissues for studies of oral peptide and nanoparticle delivery. Important differences as compared to commonly used cell culture models are identified. To investigate the maximal absorbable fraction of selected peptide delivery systems, we used a simple mechanistic computational model for scaling in vitro and ex vivo experimental permeability data to human in vivo intestinal permeability.

## 2. Peptide drugs

The number of peptide drugs in preclinical development and undergoing clinical trials has increased in recent years [4]. This is partly explained by the larger size of peptides compared to conventional drugs. The greater size allows for interaction with targets with shallow and or extended binding pockets that are not usually accessible to small molecular drugs. Such targets include intracellular protein–protein interactions, which are implicated in many human diseases. A requirement for interaction with such intracellular targets is that the peptide drug can permeate the cellular plasma membrane and reach the intracellular compartments. In general, this does not seem to be the case, since the vast majority of peptide drugs have extracellular targets [4]. Further, they are administered by parenteral routes, suggesting that they cannot permeate cellular barriers such as the epithelia underlying our mucosal surfaces. If peptide drugs could be modified or formulated to permeate epithelial barriers (in particular the intestinal barrier after oral administration) and later, the plasma membranes of the target cells, tremendous therapeutic advantages would result.

So what are the limits with regard to the molecular properties that would allow for oral administration of peptide drugs? Doak et al., recently investigated this issue [5]. An excellent and detailed analysis was performed of all drugs and clinical candidates outside the rule of five, including 226 oral drugs of various types. The authors observed

that a chemical space far beyond the rule of five allows for oral administration of (peptide) drugs. Thus, a molecular weight of up to 1000 D, a ClogP up to 10 and a 2D polar surface area (PSA) up to 250 Å<sup>2</sup> may still allow for oral administration. The number of hydrogen bond donors could, however, only be extended to 6. The 2D PSA limit of 250 Å<sup>2</sup> is much higher than the originally proposed 140 Å<sup>2</sup> [6]. Interestingly, this could be explained by 3D PSA analysis showing that the formation of flexible interactions, such as intramolecular hydrogen bonds and dipole–dipole interactions reduced the polar surface area to values below 140 Å<sup>2</sup>.

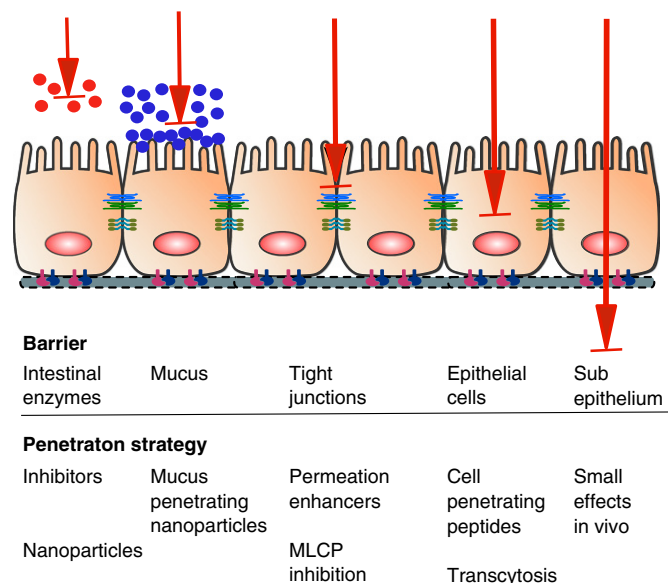
In summary, the analysis of Doak et al., extends the chemical space for oral peptide delivery beyond the rule of five [5]. However, it is clear that for the many peptide drugs larger than approximately 1000 D, other approaches such as optimized delivery systems are required to enhance peptide permeation across the intestinal barriers and the plasma membranes of the target cells. In the following, we will review these barriers, present ways to circumvent them and then focus on predictive models for the assessment of peptide delivery across the intestinal barrier. For the sake of clarity, we use the expression peptide for all peptides and proteins that require additives or delivery systems, e.g. absorption enhancers, or nanoparticles to be absorbed via the oral route.

## 3. The barriers

On its way through the gastrointestinal tract, any drug will encounter a series of barriers before it reaches the capillaries in the subepithelial tissue (Fig. 1). The main barriers are the gastric and intestinal milieu, the mucus barrier, the tight junctions blocking paracellular passage, the epithelial cells of the gastrointestinal tract, and finally the subepithelial tissue. Here we summarize the hurdles that an orally delivered peptide drug must overcome before being absorbed. Examples of interesting strategies that have been attempted to overcome the barriers are presented.

### 3.1. The gastric and intestinal digestive environment

After oral intake, a peptide drug will encounter a series of digestive enzymes whose purpose is to degrade macromolecules into absorbable nutrients [7,8]. The enzymes involved in digestion of food constituents are well studied and have been comprehensively reviewed elsewhere [9–11]. Data on enzyme expression levels and tissue localization can be found in the Human Protein Atlas ([www.proteinatlas.org](http://www.proteinatlas.org)) [12,13]. Digestive enzymes in the saliva are mixed with the oral drug already in the mouth. The enzymes lingual lipase (EC 3.1.1.3) and amylase (EC 3.2.1.1) are secreted in the saliva and start the breakdown of fats and sugar during mastication. The zymogens pepsinogens A (EC 3.4.23.1) and pepsinogen C (EC 3.4.23.3) are then added to the mix in the stomach and activated to the proteases pepsin A and gastrin by hydrochloric acid. Trypsinogen (EC 3.4.23.18) and chymotrypsinogen (EC 3.4.21.1) are added to the chyme with the pancreatic fluids that are secreted into the duodenum after food intake in response to, among other



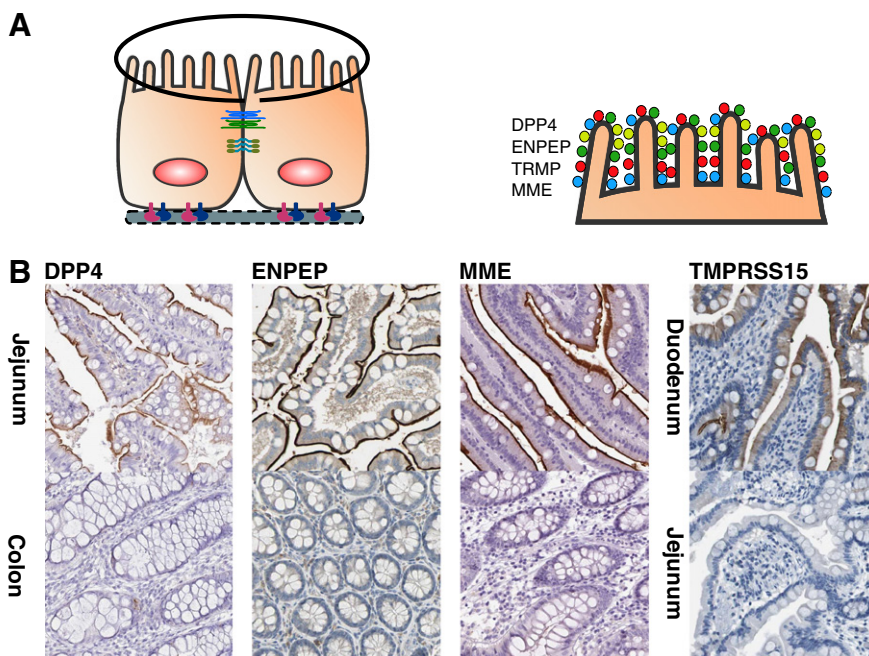
**Fig. 1.** The gastrointestinal barriers to oral peptide delivery. The drawing shows the main barriers to oral delivery of peptide drugs: the gastric and intestinal enzymes, the mucus barrier, the tight junctions, the intestinal epithelial cells lining the gastrointestinal tract, and the subepithelial tissue. Strategies to overcome these barriers are presented below the graphics.

things, cholecystokinin (CCK) release from the duodenal lining. These zymogens are activated into the mature proteases trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) by enteropeptidase (transmembrane protease, serine 15, TMPRSS15, EC 3.4.21.9) expressed in the duodenal epithelium. Pancreatic lipase (PNLIP, EC 3.1.1.3), elastases (a group of proteases, EC 3.4.21.36/70/71) and amylase are also secreted with the pancreatic fluids. The presence of high concentrations of proteases in the gut lumen is clearly a significant challenge for oral peptide delivery.

Bile is added to the chyme in the duodenum in response to food intake. Lipids stimulate the release of CCK from epithelial cells in the duodenum leading to gall bladder contraction and secretion of pancreatic fluid. Drug formulations or nanoparticles containing a lipid phase will start to be digested at this stage and will be disrupted by lipase and bile salts, respectively. It has been shown that solid lipid nanoparticles as well as mixed micelles are efficiently digested in the mouse intestine limiting the absorption of intact nanoparticles and micelles over the intestinal wall [3].

In addition to the luminal enzymes, the enterocytes of the small intestine express many digestive enzymes in the microvilli of the brush border membrane and within the glycocalyx. Peptidases with high expression in enterocyte microvilli include, but are not limited to dipeptidyl-peptidases 3 and 4 (DPP3, EC 3.4.14.4 and DPP4, EC 3.4.14.5), glutamyl aminopeptidase (ENPEP, EC 3.4.11.7), membrane metallo-endopeptidase (MME, EC 3.4.24.11), transmembrane protease serine 4 (TMPRSS4, EC 3.4.21.-), and transmembrane protease serine 15 (TMPRSS15 EC:3.4.21.9) (Fig. 2).

Caco-2 cell monolayers (a model commonly used to predict intestinal permeability) do not express the majority of these proteases. For instance, TMPRSS4 and DPP4 are found in Caco-2 cells at modest expression levels [14]. Despite the relatively weak expression in Caco-2 cells, both DPP4 and serine dependent peptidases (such as TMPRSS4) have been shown to digest small peptides during their permeation of a Caco-2 cell layer, and this digestion is likely to be even more efficient in vivo [15,16]. Indeed, in Ussing chamber permeability experiments with rat ileal and colonic tissues it was observed that the comparatively metabolically stable vasopressin analogs lysine vasopressin and desmopressin were enzymatically degraded to a major extent during passage of the intestinal tissue [17]. The transit time of the small intestine is rather constant and 2–4 h is the common timespan. The transit time in the colon is much longer (up to 24 h) [18–20]. For more information on GI transit times see [18,21]. Like other physiological parameters, gastrointestinal transit times display large species differences where rodent gastrointestinal transit times are much smaller than human [21]. In rat, jejunal, small intestinal, and total gastrointestinal



**Fig. 2.** Expression of brush border peptidases in the human intestinal epithelium. A. Drawings of intestinal epithelial cells with microvilli that express many peptide degrading enzymes. B. Immunohistochemical staining of selected peptidases is shown. Peptidases with high expression in enterocyte microvilli include dipeptidyl-peptidases 3 (DPP3), glutamyl aminopeptidase (ENPEP), membrane metallo-endopeptidase (MME), and transmembrane protease serine 15 (TMPRSS15). DPP3, ENPEP, and MME brush border peptidases are found at high levels in the jejunum, but not in the colon. The enteropeptidase TMPRSS15 is found in the duodenum but not in the jejunum. The immunostained sections are adapted from the Human Protein Atlas ([www.proteinatlas.org](http://www.proteinatlas.org)) [12,13].

transit times of 80 min, 3 h, and 11 h, respectively, have been reported [22–24]. Regardless of species a peptide drug that is poorly absorbed is exposed to peptide degrading enzymes for a significant amount of time.

Experience from conventional formulations shows that the peptide degrading acidic environment in the stomach can be avoided by enteric coated formulations [25,26]. Loading of the peptides into a nanoparticle can provide protection during the passage of the gastrointestinal tract allowing delivery of the peptides to or near the enterocytes of the intestinal wall. Conjugation of peptides with, e.g. lipids into lipopeptides [27], can also protect peptides from degradation, as can the addition of enzyme inhibitors [17]. In the latter case, dilution of the inhibitor in the luminal contents upon release may be a problem. It is noteworthy that the few orally administered peptides on the market (cyclosporin A and desmopressin) or under new drug application (NDA) review by the US Food and Drug Administration (FDA) (octreotide) are cyclic peptides, more specifically macrocycles [25]. Macrocycles stabilize peptides against enzymatic degradation, and the stabilizing effect of cyclization has been exploited to develop cyclic peptide prodrugs [16].

### 3.2. The mucus barrier

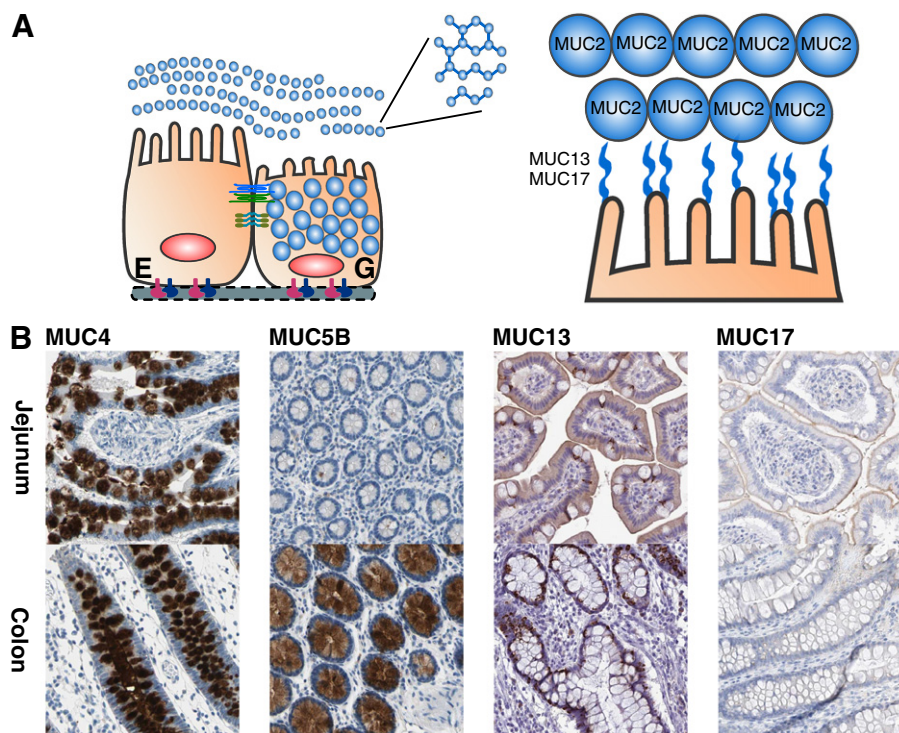
#### 3.2.1. Mucus barrier structure

The first physical barrier to absorption in the gastrointestinal tract is the mucus barrier, a hydrogel layer composed of large glycoproteins, predominantly of the mucin family [28–31]. In the small intestine MUC2 is the main mucin secreted from goblet cells, while these cells also secrete MUC5B in the colon (Fig. 3) [28,32–34]. Mucus production amounts to an average of 1 kg/day in an adult human. The mucus layer in the human intestine ranges from 10 to 100–200  $\mu\text{m}$  thick (jejunum to colon) and consists of an outer, loosely adherent layer, and an inner, thinner, and more strongly adherent layer. The inner, strongly

adherent layer has been estimated to be 7  $\mu\text{m}$  thick in the colon, but its thickness has not to our knowledge been determined in the human small intestine [35,36]. The outer loosely attached layer is thin in the small intestine (10  $\mu\text{m}$ ) but thicker in the colon (40–100  $\mu\text{m}$ ) [28, 35–37]. The mucus barrier shows large species variations, with the rat intestinal mucus layer being tenfold thicker or more in all segments of the intestine compared to the thickness in humans [36–38]. Both mucus layers consist of stacked, parallel sheets of mucin molecules covering the intestinal surface on top of and between the villi in the small intestine [34,39]. Some structural features of mucus have been identified. In mucin-2 (MUC2) based mucin-sheets each mucin-2 protein binds to three neighbors, creating a hexagonal mesh [29,34,40].

Closest to the epithelium is the 0.5  $\mu\text{m}$  thick glycocalyx, comprised of polysaccharides bound to membrane anchored mucin proteins, MUC 1, 3, 12, 13 and 17 in the human jejunum and colon [12,32,41] (Fig. 3). In both tissues, MUC13 and 17 seem to exhibit the strongest expression. In addition, the glycocalyx of the goblet cells contains MUC4 that is exclusively expressed by these cells [12,42]. Using transmission electron microscopy, it has been shown that the glycocalyx is pronounced over enterocytes, but of much lesser thickness over microfold cells, M-cells which are epithelial cells covering the lymphoid follicles in the intestine [43,44]. In addition to mucins, the mucus layers also contain large amounts of enzymes with the capacity to break down sugars, lipids and proteins to provide digested nutrients for absorption by the enterocytes (see Section 3.1). These enzymes pose a risk of digestion for peptide drugs entering the mucus layer.

The role of the mucus layer is to lubricate the passage of chyme, to protect the epithelium from mechanical damage and to bind pathogens and hinder them from reaching the epithelial cells [28]. The small intestine is a rather sterile environment, but in the colon, gut flora typically resides in the outer mucus layer down to the surface of the inner, strongly attached mucus layer [35,38]. While the glycocalyx restricts



**Fig. 3.** Expression of mucins in the human intestine. A. Drawings of intestinal enterocytes (E) and goblet cells (G). Mucins of the glycocalyx and mucins secreted by goblet cells that form the extracellular mucus barrier are shown. Parallel sheets of secreted mucins cover the villi of the small intestine. The sheets are made up of mucin aggregates, in the jejunum each Mucin-2 (MUC2) bind to three other mucin proteins, creating a hexagonal mesh. In the jejunum Mucin-2 (MUC2) is the major secreted mucin while in the colon Mucin-5B (MUC5B) is secreted as well. The structure of Mucin-5B containing mucus has not been determined. B. Expression of selected mucins in the jejunum and colon. Mucin-4 (MUC4) staining is seen exclusively in goblet cells. Mucin-5B (MUC5B) is found in the colon only. Mucin-13 (MUC13) is found in the glycocalyx of the enterocytes in both jejunum and colon. Mucin-17 (MUC17), which is also part of the glycocalyx, shows higher expression in the jejunum than in the colon. The immunostained sections are adapted from the Human Protein Atlas ([www.proteinatlas.org](http://www.proteinatlas.org)) [12,13].

direct contact of nanoparticles and microorganisms with the cells of the villus epithelium, the thin glycocalyx over M-cells of the lymphoid follicles presents a weaker barrier [43,44].

Caco-2 cells are a common model of the human intestinal epithelium, but a drawback associated with this cell model is that they lack a mucus layer. Caco-2 cells only express detectable levels of the glycocalyx mucins MUC13 and MUC17 [14]. Further, as for M-cells, the glycocalyx of Caco-2 cells is thinner than in normal enterocytes, which is probably related to their tumor origin [45]. Frey et al., concluded that a comparably good uptake of gold nanoparticles by Caco-2 cells compared to that in intestinal tissues might be due to this thin glycocalyx [44]. This is supported by the observation that intestinal epithelial M-cells in lymphoid follicles, that have the capacity to transcytose nanoparticles, have a less pronounced glycocalyx [43]. Goblet cell clones isolated from the colonic epithelial cell line HT-29 cells secrete MUC2, 5AC (normally found in the stomach, but not in the intestines), and colonic MUC5B, and, additionally, show weak expression of MUC1, 3 and 4 [46–48].

### 3.2.2. Mucus interactions

The mucus binds nanoparticles and proteins via hydrophobic interactions [49]. Charged groups of the mucin proteins can also interact with charged particles and immobilize them in the mucus [50]. The charge density in the mucus mesh will depend on local ionic strength and pH, and will thus be influenced by the chyme content in the intestine. The ionic strength, ionic composition, and pH have all been shown to vary significantly depending on the location in the intestine, feeding status and meal contents. Osmolality and ionic strength can fluctuate from hypotonic to isotonic to hypertonic within short distances in the gut after a meal [51]. Under hypertonic conditions, charge interactions between mucus and particles will be partially shielded by ions in the fluid, reducing interactions below the levels seen in hypotonic fluids [50]. Particle interactions with the mucus will depend partially on, among other factors, feeding state. It has recently been demonstrated that postprandial levels of calcium, bile acids and lipids lead to a denser and more impenetrable mucus, presumably by influencing interactions between mucin molecules affecting mucin–mucin binding and mucus mesh architecture [52]. In addition, dietary and pharmaceutical polymers such as pectin and PEG can compress mouse colonic mucosa by up to 80%, tightening the mucus structure [53].

Immobilization of peptides, particles or pathogens in the outer loosely adherent mucus layer will lead to rapid clearing from the gastrointestinal tract as this mucus layer is shed. Small solutes, such as nutrients do, however, diffuse unimpeded through the mucus layer [35]. It has been shown that densely charged but overall neutral hydrophilic particle surfaces exhibit limited interactions with mucus [54]. Indeed, for many virus capsids, the diffusion rate through native cervical mucus approaches the diffusion rate in phosphate buffered saline solution (PBS) [54,55]. Olmsted et al., showed that for particles with densely charged, net neutral, hydrophilic surfaces, the mucus mesh size was approximately 100 nm [54].

Building on these findings, mucus penetrating particles (MPP) were developed [56,57]. By decorating the surface of nanoparticles with a dense layer of low molecular weight PEG-chains, particles were created with weak mucus interactions that are capable of rapid diffusion through the cervical mucus layer. When the density of PEG-chains was decreased, the particles were trapped in the mucus layer. Further, nanoparticles covered with longer PEG-chains did not penetrate the mucus layer, probably due to entanglement between the longer PEG-chains and the mucus mesh. The MPPs were found adjacent to the epithelial cell layer while conventional nanoparticles were immobilized in the outer regions of the mucus layer [57]. Many soluble proteins also display a hydrophilic surface, leading to limited interactions with mucus proteins. Nanoparticles covered with serum albumin exhibit weak, repulsive mucus interactions due to the negative charge of the albumin protein [58]. In theory, nanoparticle binding to the mucus

surface layer might be a viable strategy for the delivery of small molecules and stable peptides allowing concentrated, controlled release of the peptide in the vicinity of the enterocyte. In practice, the loosely attached outer mucus layer and peptidases of the intestine will be a significant barrier to this approach. MPPs are therefore an attractive strategy, but their value after oral administration in humans remains to be proven.

The densely charged, hydrophilic surface of MPPs is detrimental to membrane permeation and entry into the epithelial cell. To address this problem, a multifunctional MPP was developed that first shed its outer mucin-penetrating shell as it passed the mucus layer [59]. Shedding of the shell exposed the inner core structure of the nanoparticle composed of a complex of the cell penetrating peptide (CPP) penetratin and insulin. This construct led to a 60% blood glucose lowering in rats after administration of a comparably high dose of 75 IU/kg. While this construct demonstrated proof of concept, its advanced multicomponent structure might make it impractical for clinical investigation.

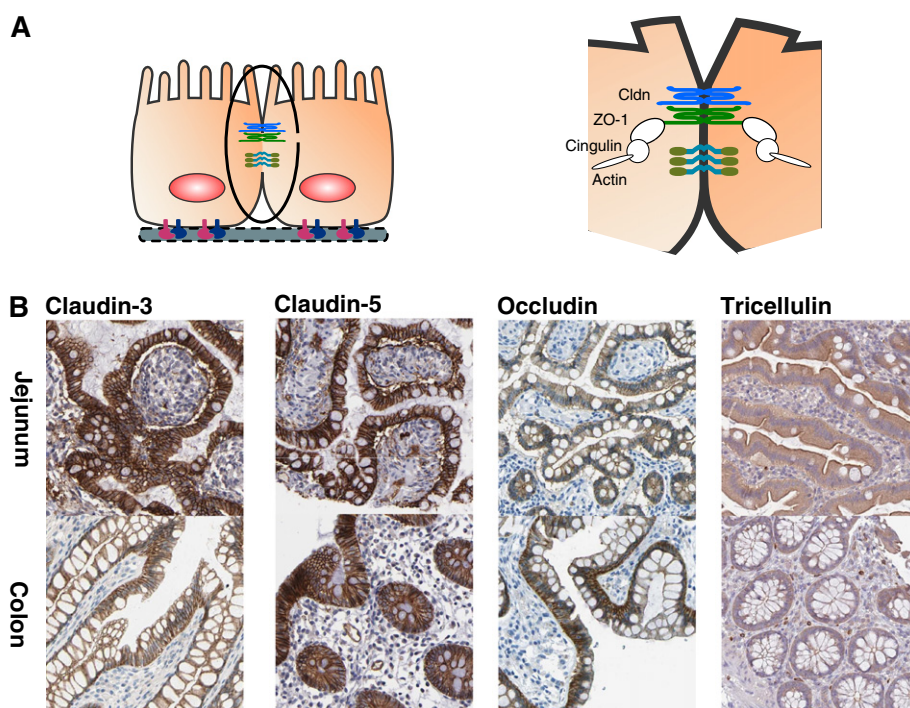
### 3.3. Tight junctions

#### 3.3.1. Tight junction structure and function

Tight junctions are seals between adjacent epithelial cells restricting paracellular flux of water, ions and solutes [60,61]. The tight junctions are localized in a ring towards the apical pole of the cell. The junctional strands separate the basolateral and apical membranes of the epithelial cells and are instrumental in maintaining epithelial and endothelial tissue barriers as well as cell polarity.

The junctions are composed of branching strands which are themselves composed of rows of transmembrane proteins with extracellular domains, predominantly of the 27-member claudin (CLDN) family and occludin (OCLN) [62,63]. The extracellular domains of these proteins form loops that bind to the corresponding loops of adjacent cells, creating a seal between the two cells [64,65]. At the junctions between three adjacent cells, a third protein, tricellulin (MARVELD2), forms a junction [60,61]. Claudins are found in various combinations in different tissues and there is strong evidence that these proteins are responsible for the paracellular barrier functions in various tissues, including the intestine [62,63]. The intracellular domains of the claudins are linked to peripheral scaffolding proteins such as ZO-1 (zonula occludens 1). ZO-1 is in turn connected to cytoskeleton components such as actin, myosin, and microtubules via linker proteins. Phosphorylation of several of these intracellular proteins affects the junctional complex and hence regulates the tightness of the paracellular barrier, a feature that has been exploited to enhance the delivery of peptides (see Section 3.3.2. below) [66,67].

Claudins are classified into two groups – barrier-forming and pore-forming, the latter controlling the selective passage of small solutes over the epithelium [63]. Of the claudins present in the jejunum, claudin-2 and claudin-15 (CLDN2 and CLDN15) are considered pore-forming (Fig. 4) [68]. Claudin-2 pores allow permeation of  $\text{Na}^+$ , some other cations, and  $\text{H}_2\text{O}$ ; while Claudin-15 pores are permeable to  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Li}^+$ , but not  $\text{Cl}^-$ . The  $\text{Na}^+$  leak through the tight junction pores is required for normal function of sodium-dependent nutrient uptake such as glucose and amino acids via transport proteins, e.g. the sodium-dependent glucose transporter 1 (SGLT1, SLC5A1) [69]. When Claudin-15 is knocked out in mice, the intestinal lumen is quickly depleted of  $\text{Na}^+$ , impeding glucose absorption [69]. The opening of the  $\text{Na}^+$  pathway is induced by SLC5A1-mediated glucose uptake in a process involving activation of myosin light chain kinase (MLCK), phosphorylation of myosin light chains (MLC), and a controlled tightening of the perijunctional actin-myosin ring resulting in an increased permeability of sodium and small solutes through the tight junction [61]. It is noteworthy that this post-prandial opening of tight junctions is compensated by the food induced strengthening of the mucus barrier discussed in Section 3.2.1 [52].



**Fig. 4.** Expression of tight junction proteins in human intestinal tissues. A. Drawings of intestinal epithelial cells, the location and organization of tight junctions proteins connecting the tight junctions with the perijunctional actin ring are shown. B. Expression of selected tight junction proteins in the jejunum and colon. Claudin-3 (CLDN3), claudin-5 (CLDN5), and Occludin (OCLN) show intense basolateral staining in both jejunal and colonic epithelia. Tricellulin (MARVELD2), which is normally restricted to tricellular junctions, is stained on the apical side of the cells. The more wide-spread staining than expected of the tissue section could be a result of varying tissue quality and overstaining. The immunostained sections are adapted from the Human Protein Atlas ([www.proteinatlas.org](http://www.proteinatlas.org)) [12,13].

Interestingly, tight junction protein expression can also be regulated by food constituents, at least under *in vitro* conditions. For instance, naringenin, a compound found at high concentration in grape fruit juice and to a lesser extent in other citrus fruits has been shown to increase the expression of claudin-1 and 4 and to tighten the junctions in Caco-2 cells [70]. The pore pathway can also be regulated by cytokines [71]. For example IL-13 leads to increased transcription of claudin-2 [71]. Stronger inflammatory stimuli such as TNF- $\alpha$  can lead to a stronger tightening of the actin-myosin ring leading to the opening of a leak pathway allowing permeation of larger solutes, even macromolecules [72–74]. This leak pathway is also associated with occludin internalization and a general weakening of the tight junction barrier [72,75]. The leak pathway is involved in diarrhea after bacterial infections in the gut and may be too difficult to control to be of interest for enhanced peptide delivery [61,72].

The individual claudin concentration and composition of the tight junctions differs along the intestine. This is reflected in permeability studies, using hydrophilic paracellular marker molecules, whose permeabilities decrease from the small intestine to the colon. The human protein atlas combined with proteomic studies, and other expression studies show that claudins 1, 2, 3, 4, 5, and 15 are expressed in the human jejunum, while claudins 1, 3, 4, 7, 8, 12, and 15 are found in the human colon, and Caco-2 cells, finally express claudins 1, 2, 3, 4, 15, and 17 (Fig. 4) [12,14,32,76–78]. The notable absence of claudin-5 in Caco-2 cells distinguishes it from human jejunum, while the absence of claudin-8 is a deviation from the colon phenotype.

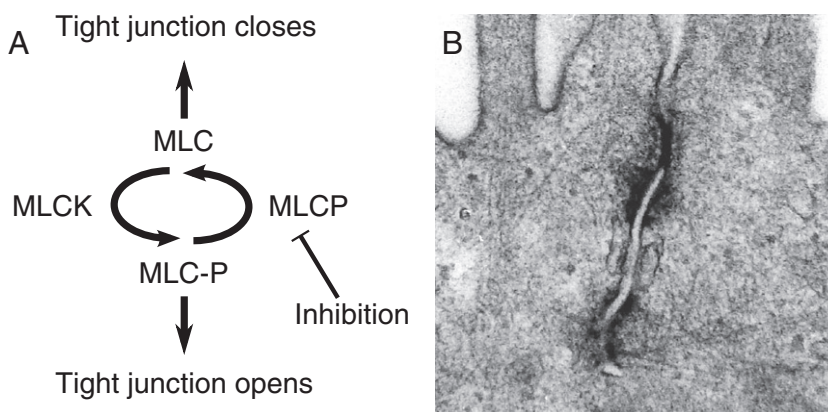
Interestingly, selective suppression of claudin-5 regulates the permeability of the blood–brain–barrier in mice and was applied to reduce brain oedema after acute brain injury [79]. Since claudin-5 is expressed in human jejunum, it could be tempting to investigate this mechanism in Caco-2 cells with the aim of improving peptide delivery. Such an investigation would be meaningless, however, owing to the lack of claudin-5 in these cells. This hypothetical example underscores the importance of prior knowledge of the differences between cellular models

and primary human tissues before initiating functional studies. Previously, such information has been difficult to obtain, but global omics data are now rapidly becoming generally available [14,32]. This will be very helpful in understanding old and discovering new mechanisms of tight junction regulation for enhanced peptide delivery.

### 3.3.2. Tight junction modulation for oral peptide delivery

Modulation of tight junction permeability has often been attempted as a means for peptides or nanoparticles to cross the epithelium. To this end, two types of strategies have been adopted, a seemingly less controlled tight junction modulation, creating leaks that allow the passage of large molecules, and a more controlled tight junction modulation. In the former case, surface active so-called absorption enhancers have been used. Familiar examples are medium chain fatty acids and their derivatives. While these agents cause reversible leaks in tight junctions within narrow concentration intervals *in vitro*, these levels are difficult to maintain after oral or local administration *in vivo* [25,80–82]. At higher concentrations, many of the surface active enhancers also interact with cell membranes, causing irreversible damage to the intestinal epithelium. Notably, the less surface active shorter medium chain fatty acid sodium caprylic acid (C8) is added in an oral formulation of the macrocyclic peptide drug Octreotide, which is under NDA review by the US FDA [25].

A better controlled tight junction modulation seems to be a more attractive way than permeation enhancers to increase paracellular peptide permeability. As indicated above, myosin light chains (MLC) are connected to the peripheral scaffold proteins of the tight junctions. MLC are phosphorylated by myosin light chain kinase (MLCK) which leads to tight junction opening [61,67]. This opening is then reversed by dephosphorylation catalyzed by myosin light chain phosphatase (MLCP) (Fig. 5) [83]. In contrast to the strong effect on the leak pathway created by TNF- $\alpha$ , or certain absorption enhancers when used at high concentrations, myosin phosphorylation via MLCK seems to be more controllable and leads to tight junction opening without occludin internalization [84,85]. To exploit this finding, several MLCP inhibitory



**Fig. 5.** Tight junction modulation. A. Schematic drawing of tight junction modulation redrawn from [66]. Phosphorylation of myosin light chains (MLC) by myosin light chain kinase (MLCK) leads to contraction of the actin–myosin ring and tight junction opening. MLC-P dephosphorylation by MLC phosphatase (MLCP) reverses the process. Inhibition of MLCP tilts the balance towards tight junction opening. B. Electron micrograph of a Caco-2 epithelial tight junction connected to the actin–myosin perijunctional ring ( $\times 11000$ ).

peptides were developed [66]. Peptides giving rise to different degrees and kinetics of tight junction modulation were identified. Injections of these different peptides together with 30 IU/kg insulin into the intestinal lumen in rats resulted in a significant lowering of the blood glucose in the range of 30–50%. In untreated animals, fluorescently labeled insulin could be visualized along the epithelial brush border, and after treatment with MLCP inhibitory peptides, the labeled insulin could be seen penetrating between enterocytes and into the subepithelial tissue. It was calculated that the most efficient MLCP inhibitory peptide led to an insulin bioavailability of 3–4%, demonstrating that tight junction modulation is a promising strategy for intestinal peptide delivery. Rather high concentrations of the peptides and also of insulin were required for effective modulation and it remains to be shown if this approach holds promise when scaled up to larger species. It is also likely that the pathway opened by controlled tight junction modulation is too narrow to allow passage of nanoparticles.

### 3.4. Intestinal epithelial cells

Intestinal epithelia of the small intestine and colon present a large absorptive surface that mainly consists of absorptive enterocytes and, to a lesser extent, of mucus secreting goblet cells. From studies in rodents, the fraction abundance of goblet cells has been estimated at 10–20%, increasing towards the colon [86,87]. Less than 1% of the epithelial cells are enteroendocrine cells secreting hormones such as Glucagon-like peptide-1, GLP-1, in response to stimuli in the intestinal lumen [88,89]. Dendritic cells of the immune system are also found within the epithelium, but they are scarce [90]. Microfold cells, M-cells, are a rare type of epithelial cell found in lymphoid follicles in the intestinal tract [91]. Larger follicles and surrounding tissue are referred to as Peyer's patches. M-cells have received much attention as a potential route of nanoparticle or peptide delivery as a result of their comparatively high rate of transcytosis [92,93]. Most of these studies have been performed in rodents. The prevalence of M-cells is much lower in man and estimates of M-cell nanoparticle uptake in rodents may, therefore, not translate to humans [94].

The total length of the human small intestine is 6–7 m, 2.5 m of which belongs to the jejunum. The internal surface of the jejunum is very large and convoluted. This is the result of large transversal folds, the folds of Kerckring, and the villi, 1 mm long, fingerlike or leaf-like projections covering the intestinal epithelium. Villi are longer and more densely spaced in the jejunum than in other parts of the small intestine. The apical membrane of the absorptive enterocytes is covered by densely packed microvilli, further amplifying the surface area of the intestine. The total surface area of the small intestine is commonly

estimated to be 250–300 m<sup>2</sup>, although recent studies suggest that the area is smaller, approximately 30 m<sup>2</sup> [95,96]. The discrepancy is caused by the problems inherent in determining the length, diameter, and folding of the small intestine that varies with the tonus of the underlying muscle layers. Measurements post mortem and in living subjects show large differences, making area estimates uncertain. The surface of the colonic mucosa is much smaller, due to the lack of villi, and is commonly estimated at 1–2 m<sup>2</sup> [96]. Fig. 6 shows the appearance of intestinal villi and microvilli of absorptive cells.

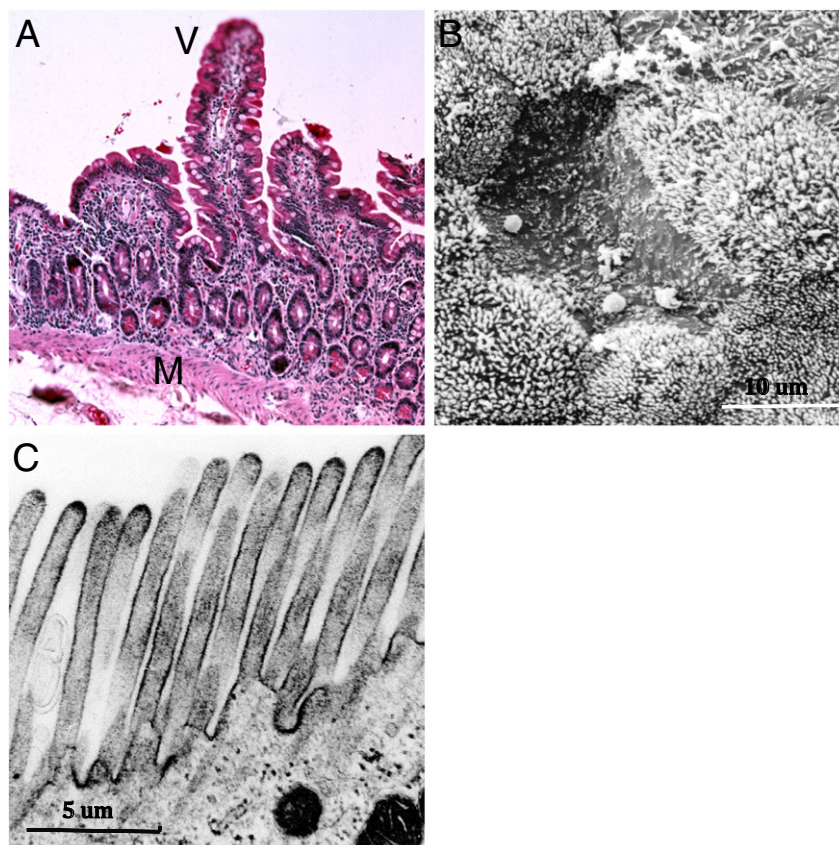
Small lipophilic peptides can, depending on molecular properties, distribute into the phospholipid bilayer and thus pass an epithelial cell by simple diffusion [5]. As indicated in Section 2, peptide drugs are, with some exceptions, generally too large and polar to penetrate the cell membrane. Peptide transporters such as peptide transporter 1 (PEPT1, SLC15A1) show a large capacity for the transport of di- and tripeptides, but most peptide drugs are much larger [97,98]. Transport proteins suitable for the delivery of large peptide drugs have not been identified in the plasma membranes of intestinal epithelial cells. Alternative routes across the cell membrane(s) must, therefore, be targeted.

#### 3.4.1. Cell penetrating peptides

One such route is exploited by so-called cell penetrating peptides (CPPs), of which the first discovered was the trans-activating transcriptional activator (TAT) from human immunodeficiency virus 1 [99]. CPPs are a heterogeneous collection of cationic or amphipathic peptides of 5–30 amino acid lengths. They are derived from natural proteins (e.g. penetratin) or artificial sequences (e.g. octarginin) that have the ability to disrupt and penetrate the plasma and/or endosomal membrane [100, 101].

There are about 1000 CPPs that may enter cells by means of a number of different mechanisms and that express a wide variety of toxicities [100,102]. This complexity has slowed down the application of CPPs for peptide delivery. CPPs with the ability to deliver cargo (e.g. therapeutic peptides) often enter the cell through endocytosis and subsequent lysis of the endosomal membrane [103]. Thus, a CPP-delivered therapeutic peptide will encounter additional proteolytic activity in the endosomes. CPPs have, therefore, been combined with nanoparticle encapsulated cargo. Apart from protecting the peptide, this approach has the additional theoretical advantage of keeping the CPPs sufficiently concentrated to facilitate the exit from the cell into the subcellular tissue.

Cell penetrating peptides have been incorporated into nanoparticles with promising results. A mucus penetrating nanoparticle with a CPP core was discussed in Section 3.2.2 [59]. Another nanoparticle strategy combined a CPP (penetratin) with a secretion peptide, Sec, derived from the secreted transcription factor, Engrailed, on the surface of



**Fig. 6.** Villi and microvilli of the intestine. A. Section of jejunal tissue after stripping off submucosal tissue and mounting in an Ussing chamber. The upper edge of the specimen consists of the villi (V) at the mucosal surface. The lower limit of the specimen is delineated by the mucosa muscularis (MM), on the serosal side of the mucosa (Hematoxylin-Eosin stain). B. Scanning electron micrograph of the microvilli covered surface of Caco-2 cells ( $\times 11,000$ ). Note the selective adherence of three spherical particles to a cell surface with low density of microvilli. C. Transmission electron micrograph of microvilli structure on the apical surface of Caco-2 cells ( $\times 11,000$ ). Note the absence of a dense glycocalyx.

insulin loaded nanoparticles to stimulate both entry and exit from the enterocytes [104]. The Sec peptide is a signaling peptide directing the Engrailed transcription factor to the protein export machinery in the cell [105]. The nanoparticle decorated with both peptides was shown to be significantly more effective than a nanoparticle employing only penetratin, both in *in vitro* permeability experiments in Caco-2 cells and in *in vivo* experiments. Nanoparticles decorated with penetratin alone (size 148 nm) achieved an insulin bioavailability of 9% after ileal administration of 10 IU/kg insulin in rat, while particles decorated with both penetratin and the Sec peptide (size 163 nm) increased the bioavailability of the insulin cargo to 15% [104]. However, insulin loaded into unmodified poly (D,L-lactic-co-glycolic acid) (PLGA) nanoparticles (size 160 nm) achieved a bioavailability of 5% in the same study, which seems inexplicably high. It remains to be determined whether these very promising results can be replicated in independent studies. The use of the Sec peptide to target the nanoparticle to the cells' protein export machinery to exit the cell is a similar approach to the transcytosis targeted delivery that will be discussed in the following sections.

#### 3.4.2. Endocytosis and transcytosis

The second route for peptide transport across a cellular barrier has been to target peptides or nanoparticles to a naturally occurring transcytosis mechanism present in the epithelial cells. Transcytosis is a mechanism by which polarized cells transport material from one side of the cell to the other [106]. The first step of the transcytotic route is endocytosis. Endocytosis is a general mechanism present in all cells that transport membrane proteins and extracellular material in membrane vesicles into the cell interior [107]. The mechanisms of

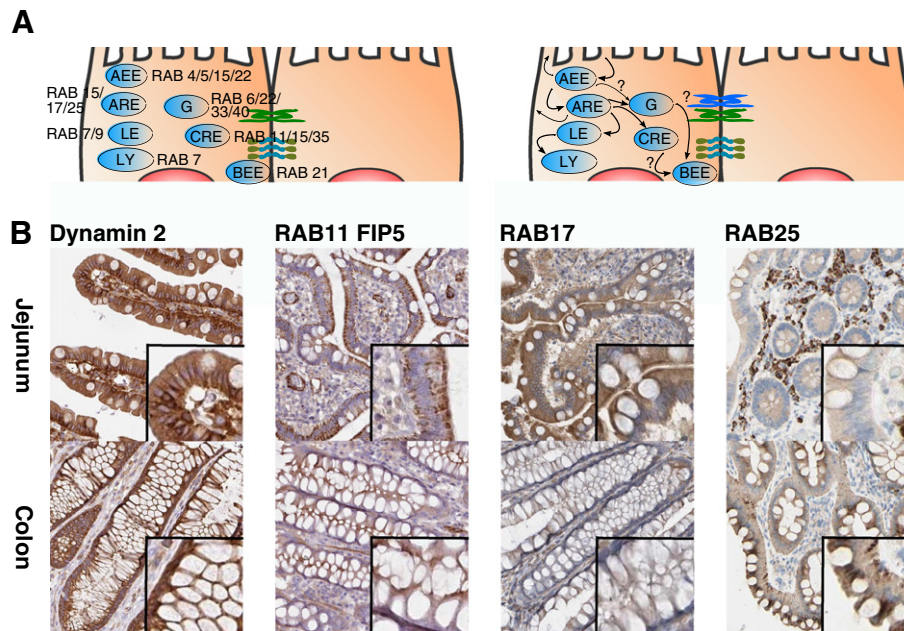
endocytosis have been relatively well studied (Fig. 7A). In contrast, the mechanisms of transcytosis are not as well understood.

Transcytosis across epithelia is mediated by specific mechanisms and is a rather rare event. Examples of transcytotic mechanisms, some of which have been exploited for peptide and nanoparticle delivery are given in Table 1.

Endocytosis is classically divided into four separate mechanisms, clathrin-mediated endocytosis, caveolae, macropinocytosis and phagocytosis. Many studies show that in addition to these pathways a number of clathrin- and caveolin-independent mechanisms exist (reviewed in [107,127]). Clathrin- as well as caveolin-dependent endocytosis involves receptor binding of macromolecules and subsequent internalization. Typically, in neither of these cases, do the endocytotic vesicles exceed 150 nm in diameter, but exceptions occur [128–130]. Macropinocytosis internalizes small droplets of liquid and solutes from the extracellular fluids [131]. The diameter of endocytotic vesicles resulting from macropinocytosis can be larger than those from clathrin and caveolin-mediated endocytosis. Phagocytosis involves the internalization of larger particles and pathogens [107]. All of these four endocytosis mechanisms are dependent on dynamin motor proteins that split the endocytosed vesicle from the plasma membrane [132,133]. It has been proposed that all four of the classic endocytosis mechanisms are involved in nanoparticle uptake in epithelia [134,135].

Caco-2 cells display comparatively low endocytotic activity compared to intestinal epithelia and also lack detectable expression of caveolins using high resolution mass spectrometry [14]. However, caveolins have been detected in differentiated Caco-2 cells by western blotting [136]. To enhance endocytotic activity in the *in vitro* model a co-culture system has been devised that induces an M-cell like phenotype in the Caco-2 cells (see Sections 3.4.4 and 4) [137,138].





**Fig. 7.** Expression of dynamin and of Rab GTPases in human intestinal tissues. A. Drawings of intestinal epithelial cells showing RAB-profiles of endosomes and organelles involved in endocytosis and transcytosis. Known and hypothesized (marked by ?) pathways of endocytosis and transcytosis are illustrated. AEE, apical early endosome; ARE, apical recycling endosome; BEE, basolateral early endosome; CRE, common recycling endosome; LE, late endosome; and LY, lysosome. B. Expression of selected proteins involved in endocytosis and transcytosis in the intestinal epithelium. Dynamin-2 is highly expressed in jejunal and colonic epithelia. The Rab interacting protein Rab11FIP5 has been implicated in transcytosis. Rab11FIP5 displays punctate staining below both the apical and basolateral membranes, expression levels being higher in the jejunum than in the colon. RAB17 and RAB25, both of which have been associated with the transcytotic process are expressed in both jejunal and colonic epithelia. The immunostained sections are adapted from the Human Protein Atlas ([www.proteinatlas.org](http://www.proteinatlas.org)) [12,13].

A problem with identifying the pathways involved in nanoparticle endocytosis is the lack of specific inhibitors. Therefore a general inhibitor of dynamin-mediated vesicular internalization, such as dynasore, that inhibits all the classical endocytosis pathways is typically used [139,140]. For specific pathways chlorpromazine is considered a selective inhibitor of clathrin-mediated endocytosis, while filipin inhibits caveolin-mediated endocytosis [141,142]. For macropinocytosis, no selective inhibitors have been identified [141].

Rab GTPases control endosome trafficking in eukaryotic cells [143]. Expression of more than 50 different Rab isoforms has been detected at the protein level in the intestinal epithelium [32]. Many Rab isoforms are specific for particular organelles and endosomes. Co-localization studies using Rab isoform specific antibodies have been used to track endocytosed and transcytosed materials. Rab GTPases act as molecular switches; their function in vesicular transport has been comprehensively reviewed elsewhere [143,144]. Below, RAB involvement in endocytotic and transcytotic pathways is briefly discussed.

After budding of the endocytotic vesicle from the apical cytoplasmic membrane, vesicles from all endocytotic mechanisms are internalized into a common pool of apical early endosomes (AEE) positive for,

RAB4, RAB5, RAB15, and RAB22. A fraction of internalized membrane proteins are recycled to the membrane from these endosomes. RAB22 is involved in vesicle transport between AEE, the trans-Golgi network and the Golgi apparatus suggesting that this protein might play a role in transcytosis (see below) [145]. A subset of internalized material is transported further into RAB11, RAB17, and RAB25-positive apical recycling endosomes (ARE) and a portion is recycled back to its original membrane domain. RAB11, RAB17, and RAB25 are closely linked to the process of transcytosis, suggesting that the recycling endosome plays a central role in this process (see below) [146,147]. In polarized epithelia, separate pools of early and recycling endosomes are thought to exist beneath the apical and basolateral membranes [148]. Basolateral early endosomes (BEE) are distinct from their apical counterparts in their expression of RAB21. Material in the ARE that is not recycled is generally transported into RAB7- and RAB9-positive late endosomes (LE) and from there to the RAB7 positive lysosome (LY). In the LE, the route to the lysosome has probably been set, and the option of transcytosis is no longer available [143]. Thus, the common pathways of endocytosis end either in the membrane of origin or in the proteolytic environment of the lysosome. Studies of nanoparticle transport in epithelial MDCK

**Table 1**

Examples of transcytosis mechanisms in intestinal epithelia. A subset of the transcytosed entities have been used for targeted peptide or nanoparticle delivery.

Class	Transcytosed entity	Ligand	Utilized for peptide delivery	Utilized for nanoparticle delivery	Reference
Receptors	Neonatal Fc receptor (FcRn, FcGRT)	IgG	Yes	Yes	[108,109]
	Polymeric immunoglobulin receptor (PIGR)	pIgA	No	No	[110]
	Lactoferrin receptor (ITLN-1)	Lactoferrin	Yes	Yes	[111,112]
	Vitamin B12 – intrinsic factor receptor (CUBN, AMN)	Vitamin B12	Yes	Yes	[113–116]
Transporters	GLUT2 (SLC2A2)	–	No	No	[117]
Bacterial toxins	Some cholera toxins	–	Yes	No	[118,119]
	Shiga toxin	–	Yes	No	[120–122]
Lipids	Sphingolipids	–	Yes	No	
	Ceramides	–	Yes	Yes	[123,124]
Other membrane proteins	Lectins	–	Yes	Yes	[125,126]

cells showed that endocytosed polystyrene nanoparticles are transported into RAB7 and RAB9 positive compartments [149]. These compartments are likely to be late endosomes and lysosomes. A minority of nanoparticles were located in RAB11 positive recycling endosomes that might have led to transcytosis [150]. Fig. 7 shows examples of Rab GTPase expression in the small and large intestine.

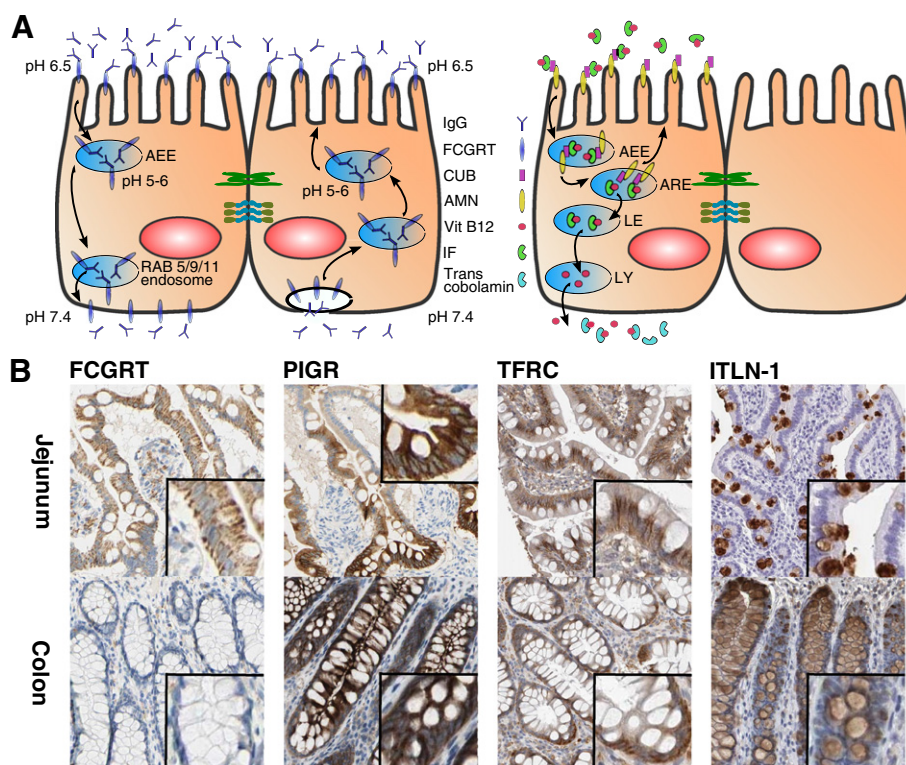
The mechanism giving rise to transcytosis is poorly understood. The process shows an inherent polarity with mechanistic differences between apical to basolateral and basolateral to apical transcytosis [151, 152]. This section focuses on apical to basolateral transcytosis. It is clear that RAB11A, RAB17 and RAB25 are involved in transcytotic transport, implicating that there is a role for apical and basolateral recycling endosomes [144,146,147]. The mechanism of endosome transfer between apical and basolateral domains remains unidentified. It has also been shown that transcytosis in MDCK cells is inhibited by Brefeldin A (BfA), an inhibitor of protein and vesicle transport from the endoplasmic reticulum (ER) to golgi apparatus, suggesting that these organelles play a role in transcytosis [153–155]. One intracellular target for BfA has been proposed to be Golgi-specific Brefeldin A-resistance guanine nucleotide exchange factor 1 (GBF1), a guanine nucleotide exchange factor involved in the formation of transport vesicles in the Golgi [156–158]. GBF1 is also involved in vesicle transport along the retrograde pathway from apical recycling endosomes, via the trans-Golgi and Golgi networks to the ER and BfA accordingly strongly modifies this pathway [159–162]. The retrograde pathway is used by several polypeptide bacterial toxins to escape degradation in the lysosome and exert their toxic effect by escaping from the ER to the cytoplasm using specialized CPPs [162–164].

Interestingly RAB11 has also been shown to be involved in targeting of endosome transport from the apical recycling endosomes to the trans-Golgi network which suggests that the retrograde pathway

between these organelles is a central step in the mechanism(s) of transcytosis [165,166]. Activation by mitogen activated protein kinase (MAPK) dependent phosphorylation of the Rab-binding protein RAB11FIP5 leads to increased transcytosis in MDCK cells [166]. These proteins are expressed in jejunal and colonic epithelia and at lower levels in Caco-2 cells [12,14,32]. RAB11FIP5 shows distinctively higher expression levels in jejunal than in colonic epithelium. In both tissues, the protein shows a distinct subcellular localization (Fig. 7B). The protein is found in punctate stains close to both apical and basolateral membranes that could be identical to recycling endosomes.

### 3.4.3. Targeting to transcytosed receptors

An example of a protein transcytosed across the intestinal epithelium is IgG, which is transported from the gut lumen via the neonatal Fc receptor (FcRn, FCGRT) [167,168]. In humans “neonatal Fc receptor” is a misnomer as the receptor is expressed in the intestinal epithelium throughout life. In rodents the receptor expression declines rapidly after weaning. In neonatal rats FCGRT is highly expressed in the jejunal epithelium where it imports milk derived IgG from the gut lumen back into the circulation. In the slightly acidic microclimate (pH 6.5) created by the proton gradient in the jejunal mucus layer, the FCGRT binds the Fc-portion of IgG with high affinity [169]. The receptor is then transcytosed with the assistance of a unique sorting motif [170], and releases its bound antibody at the neutral pH of the subepithelial tissue (Fig. 8A). The transcytosis of FCGRT has been studied in considerable detail in the neonatal rat small intestine [152]. The translocation of the receptor from the apical to the basolateral pole of the enterocyte was found to take place in a RAB5, RAB9 and RAB11 positive endosome, adding to the complexity of the transcytotic process. RAB9, like RAB11, is known to be involved in endosome trafficking to and from the trans-Golgi network [143,144]. It has been proposed that the



**Fig. 8.** Expression of receptors undergoing endocytosis or transcytosis in the human intestinal epithelium. A. Drawings of intestinal epithelial cells illustrating the mechanisms of IgG and vitamin B12 transcytosis in the intestinal epithelium. Apical to basolateral transcytosis of IgG is better understood than the process of IgG secretion via FCGRT. B. Expression of selected intestinal receptors in the intestinal epithelium. The neonatal IgG receptor, (FCGRT) is highly expressed in jejunal and to a lesser extent in colonic epithelia. The polymeric IgA receptor (PIGR) is highly expressed in both the jejunum and the colon, but is mainly found in the crypts. The transferrin receptor (TFRC) shows a strongly polarized expression in the basolateral membrane. The lactoferrin receptor (ITLN-1) is predominantly expressed by goblet cells. The immunostained sections are adapted from the Human Protein Atlas ([www.proteinatlas.org](http://www.proteinatlas.org)) [12,13].

pathway via FcRn contributes to the monitoring of the gut lumen contents by the immune system, and that it might be involved in both defense against pathogens and in induction of tolerance to food antigens [167,171,172].

Few human studies of peptide delivery via transcytosed receptors have been performed, examples in the review will therefore by necessity derive from *in vitro* experiments and small animal species.

Perhaps the most successful use of receptor-mediated transcytosis for peptide delivery has been via the FCGRT [109]. Pridgen and co-workers decorated insulin loaded nanoparticles (mean diameter 57 nm) with IgG Fc-regions. This resulted in efficient oral delivery of insulin to wild-type mice, with a reduction of blood glucose of 45% in the mice after oral insulin administration of 1.1 IU/kg. In contrast, knock-out mice negative for FCGRT showed no reduction in blood glucose after oral administration of insulin loaded Fc-nanoparticles, demonstrating the specificity of the uptake mechanism. Importantly, the administered dose was much lower than in previous examples, involving tight junction manipulation and CCPs, respectively and corresponds to a normal, daily, human subcutaneously injected dose. This suggests that the bioavailability of the orally administered insulin must be comparably high. It will be interesting to see whether these promising results can be reproduced in larger animal species. Fc-decorated nanoparticles or Fc-fusion proteins have also been found to cross the lung epithelium *in vitro* as well as *in vivo* in non-human primates and humans [173–175].

Another example of receptor-mediated transcytosis is the uptake of vitamin B12 by intestinal enterocytes. The process is illustrated in Fig. 8A. Vitamin B12 is taken up as a complex with the intrinsic factor protein (IF) in the ileum. After binding of the Vitamin B12 – IF complex to the Vitamin B12 – intrinsic factor receptor (CUBN, AMN), the receptor and ligand are endocytosed. The receptor is recycled to the membrane while the Vitamin B12 – IF complex is routed to the lysosome. Here IF is broken down and vitamin B12 is eventually released into the portal circulation, where it is bound and transported by the transcobalamin II protein [114,176,177]. The uptake of vitamin B12 is quite inefficient, and from a 5–50 µg oral dose about 1.5 µg is absorbed, possibly indicating saturation of the transport mechanism [178,179].

The vitamin B12 pathway has been explored for peptide delivery in rats. In a targeted peptide delivery study, the appetite suppressing peptide hPYY(3–36) was fused with vitamin B12 and after administration by oral gavage to rats, achieved a bioavailability of 25% [113]. In other studies, several vitamin B12 decorated insulin loaded nanoparticles were constructed, leading to oral bioavailabilities of insulin in the 5–26% range in rat (nanoparticle mean diameters of 125–370 nm) [115, 116,180]. It is interesting to note, and perhaps of concern, that, depending on the dose, a comparable or higher bioavailability was obtained with each of the vitamin B12-decorated peptide delivery systems in the small animal species than with the natural ligand vitamin B12 in man.

Another, less obvious receptor that may be targeted for transcytosis across enterocytes is the lactoferrin receptor (ITLN-1) that mediates the intestinal uptake of lactoferrin [181,182]. ITLN-1 is highly expressed in the jejunum and colon and can be found in modest levels in Caco-2 cells [12,14,32,181,183]. The lactoferrin receptor has been used for oral delivery of lactoferrin for T cell dependent tumor inhibition and for oral delivery of lactoferrin loaded nanoparticles in breast cancer treatment; both studies were conducted in mice [111,112].

Targeting oral peptide or nanoparticle delivery to a transcytosed receptor poses the risk that binding of the targeted peptide or nanoparticle can be outcompeted by the physiological ligand if the latter is present in high levels in the gut lumen. Conversely, an essential natural ligand present at low concentrations can be outcompeted by a delivery system when this is given in high amounts. One way to avoid the competition with the natural ligand could be to develop a ligand that binds to an alternative site of the receptor and still be transported. Although this approach may seem very difficult, proof-of-concept has been

provided by a transferrin-receptor binding peptide that was then bound to the surface of a nanoparticle for transport across the blood–brain barrier [184]. Unfortunately, while the transferrin receptor (TFRC) and insulin receptor (INSR) are transcytosed across the blood–brain barrier they are not transcytosed in the intestinal epithelium [185]. The transferrin receptor is predominantly localized to the basolateral membrane in enterocytes where it binds transferrin and is endocytosed [186]. Transferrin is loaded with iron in endosomes and the transferrin-receptor complex is recycled to the basolateral membrane [186]. Whether an alternative binding site can be used on another, more suitable receptor than the transferrin receptor in the enterocyte remains to be seen. A similar strategy where FCGRT-binding peptides were fused to therapeutic proteins allowing them to transcytose across MDCK monolayers has been explored [108]. Expression of transcytosed receptors in the intestine is shown in Fig. 8B.

As the number of identified receptors that are transcytosed in the intestinal epithelium is limited, other components of the apical membrane might be utilized for targeted delivery of peptides or nanoparticles. Glycosphingolipids are constituents of the plasma membrane involved in modulation of intracellular signaling [187,188]. The ceramide domain of the glycosphingolipid GM1 controls its intracellular sorting; this is also the case after endocytosis [189,190]. If the ceramide domain contains a short lipid chain, C12 or below, a fraction of the glycosphingolipid GM1 is sorted into early and recycling endosomes after addition to the apical face of the cell [190]. A fraction of the GM-1 C12-fusion is then transcytosed. If the lipid chain is longer, C18, the glycosphingolipid is mainly sorted into late endosomes and directed to the lysosomes [190,191]. Fusing glucagon-like peptide 1 (GLP1) to a short chain glycosphingolipid GM1 allowed the peptide hormone to be transcytosed over polarized epithelial MDCK cells and the nasal epithelium *in vivo* in mice [123].

#### 3.4.4. Nanoparticles and M-cell mediated transcytosis

Recently it was suggested for the first time that the body produces and absorbs endogenous nanoparticles in the small intestine [192, 193]. Phosphate and calcium ions secreted by the intestinal epithelium, will complex with calcium ions and precipitate as calcium-phosphate nanoparticles in the gut lumen. It was suggested that these endogenous nanoparticles trap antigens that are afterwards sampled by M-cells in Peyer's patches. The nanoparticles are then transcytosed to the antigen presenting cells located below the patches.

This newly discovered mechanism was proposed as a sampling mechanism from the intestinal lumen in order to induce immune responses to luminal antigens when needed and perhaps also to maintain food tolerance [93,171]. While the primary role of intestinal lymphoid tissues such as the Peyer's patches is to mediate immune responses to antigens and pathogens, these tissues also play a role in the development of oral tolerance to food antigens [171].

After transcytosis by the M-cells to the antigen presenting cells, the endogenous nanoparticles are broken down [192]. While this mechanism can be of interest for peptide delivery to the local immune system e.g. in oral vaccines, its applicability for systemic delivery remains to be shown [1,194]. Another question that remains to be answered is the capacity of the process. When studying peptide absorption across the human intestinal epithelium, it was found that tissue specimens containing Peyer's patches transported no more than twice the amount of peptides than did neighboring M-cell free epithelium [195,196]. While the physiological antigen sampling mechanism might be efficient, the Peyer's patches constitute a very small fraction of the intestinal surface. In a young adult, a few hundred patches are found in the ileum; this number then declines with increasing age [197,198]. Compared to the surface area covered by enterocytes, the area of the Peyer's patches is miniscule. The fraction of M-cells compared to other epithelial cells in the intestine has been estimated to 1 in 10 million cells [199]. The density of Peyer's patches in the intestine of common laboratory animal species is several times higher than in man [21]. This difference must

be considered to avoid an overestimation of the nanoparticle transport capacity in man.

M-cell transcytosis has often been seen as a promising route of nanoparticle delivery, particularly as M-cell transcytosis seems to bypass the lysosomes [1,200]. The efficiency of M-cell transcytosis as compared to that of the normal villous epithelium has been compared in a number of studies. In *in vitro* experiments with human intestinal tissues it was found that the permeability of RGD-peptide decorated nanoparticles was 5 times higher in tissue specimens containing Peyer's patches compared to normal villous epithelium [201]. When rat, ovine and bovine villous epithelium and Peyer's patch containing epithelium were compared, the HRP permeability across the tissue was 5-fold higher for tissue sections containing patches [202]. Uptake of *Salmonella typhimurium* bacteria was 10-fold higher in the Peyer's patches than in the villous epithelium [202].

In rat intestinal everted sacs, styrene maleic acid nanomicelles showed identical transport in jejunum and ileum, despite the higher density of Peyer's patches in the ileum [203]. The nanomicelles were absorbed to equal extents by M-cells and nearby enterocytes. In another study, uptake of polyalkylcyanoacrylate nanocapsules in M-cells was determined to be twice that of uptake in nearby enterocytes [204]. Thus the M-cell capacity for particle uptake seems on par with to ten times higher than that of the normal villous epithelium. However, since there is only 1 M-cell in 10 million enterocytes, the total capacity for nanoparticle uptake in the latter is much higher.

While the extent of Peyer's patch particle uptake seems low, few studies have been published quantifying the capacity and kinetics of the process. In one such study a dose of  $4 \times 10^8$  polystyrene nanoparticles was administered to rabbit intestinal loops with Peyer's patches [205]. Each loop contained on average 15 Peyer's patches. After 90 min of exposure nanoparticle uptake into the follicle associated epithelium of the patch and the underlying subepithelial dome was quantified. The most active patch was found to have absorbed approximately 6000 nanoparticles. If all patches had shown similar activities 1 in 4500 nanoparticles would have been absorbed, translating to a very low bioavailability. It was noted in the same study that rabbit Peyer's patches showed 10-fold higher particle absorption than mouse patches.

What is the fate of particles after transcytosis across the M-cell? This was investigated in mice where  $\beta$ -glucan microparticles were taken up by M-cells and transcytosed into the subepithelial dome of the Peyer's patch [206]. Here the particles were absorbed by a subset of dendritic cells. The particles remained in these cells until the end of the experiment, three days later. Polystyrene nanoparticles have also been found to be associated with dendritic cells up to 14 days after uptake in a Peyer's patch [207]. This suggests that absorbed particles show limited transport from the patch into the systemic circulation, but could possibly be used for pharmacological intervention in dendritic cells.

### 3.5. Subepithelial tissue

The subepithelial tissue resides below the intestinal epithelium, and can be divided into the lamina propria and muscosa muscularis. *In vivo*, this loose and highly vascularized connective tissue is not a rate-limiting barrier to the passage of small peptides [208,209]. Larger peptides, especially over 30 kDa, are preferentially transported into the lymphatic system, rather than the blood vessels [209]. In this respect, intestinal absorption and subcutaneous or intramuscular injections follow the same pattern. The cell density in the subepithelial layer is low, especially compared to the densely packed epithelial layer (see Figs. 2–8). The extracellular matrix in dense connective tissues such as cartilage can be very compact, exhibiting pore sizes as small as 60 nm [210]. However, the pore size of the extracellular matrix in the intestinal subepithelium is much larger than this and is unlikely to impede nanoparticles to any significant extent [211,212].

## 4. Caco-2 cells as an *in vitro* model system of intestinal absorption

Caco-2 is a human colon cancer cell line that, when cultured on semi-permeable filter supports, differentiates into an enterocyte-like phenotype. Since its introduction, it has become a workhorse for permeability studies in drug discovery and development as well as in the ADME (drug absorption, distribution, metabolism and excretion) sciences [213–216]. Well established protocols allow the prediction of human intestinal fraction absorbed from Caco-2 permeability data as long as intestinal drug metabolism or active transport is not significant for the investigated drug [217,218]. Caco-2 cells lack expression of the majority of the drug metabolizing cytochrome P450 enzymes found in the small intestine, limiting their usefulness for the prediction of oral bioavailability [14].

We recently quantified the proteome of filter-grown Caco-2 cells [14]. Despite its colonic origin, the Caco-2 cell line shows a proteome expression profile closer to the jejunal enterocyte. Yet, important differences exist between the Caco-2 cell line and the intestine. For instance, a lower expression of brush border peptidases (Section 3.1) and mucins was observed. Several brush border proteinases and peptidases were below the limit of detection, including dipeptidyl-peptidases 3 (DPP3), glutamyl aminopeptidase (ENPEP), membrane metallo-endopeptidase (MME), and transmembrane protease serine 4 (TMPRSS4) (and others) which are all highly expressed in enterocytes. Only DPP4 and TMPRSS15 were found at detectable levels in Caco-2 cells (Section 3.2.1). Caco-2 metabolism of peptides will therefore likely be lower compared to that in the human intestine.

Attempts have been made to compensate for the absence of a protective mucus layer in Caco-2 by co-culture with various mucus secreting goblet cell clones isolated from the human colorectal adenocarcinoma cell line HT-29 [46]. Unfortunately these two cell lines do not mix well in culture and HT-29 cells tend to grow as colonies embedded in Caco-2 cells, resulting in a discontinuous mucus layer [219–223]. Co-cultures display lower transepithelial electrical resistance (TEER, also often given as TER) than pure Caco-2 cultures and also show increased permeability of integrity markers and peptides [222]. The presence of a mucus layer has been shown to impede nanoparticle permeability in co-cultures [223]. Considering the discontinuous mucus layer it is likely that HT-29/Caco-2 co-cultures underestimate nanoparticle binding to mucus. Monocultures of goblet cell clones (Table 2) seem, depending on clone to give a better coverage and have found some application, e.g. in studies of bioadhesive delivery systems [224,225]. Importantly, the goblet cell clones will not form a mucus layer that is identical with regard to composition and structure to those found along the gastrointestinal tract. Therefore, freshly isolated mucus layers from the region of interest constitute better models [226]. In addition of having the right composition, freshly isolated mucus may retain its tertiary structure.

One aspect that has limited the usefulness of Caco-2 cells for studies of peptide delivery is the comparatively low level of endocytosis and transcytosis [137,138]. While this may be explained in part by the physiological barrier function of the intestinal epithelium, the proteome analysis showed reduced expression of proteins involved in endocytosis, such as caveolins and some syntaxins. Considering the tumor origin of Caco-2 cells, it is interesting to note that caveolin-1 can act as both a tumor suppressor and as an oncogene depending on context [227–229]. In the early stage of colorectal cancers, caveolin-1 suppresses tumor progression, while over-expression of caveolin-1 reduces the tumorigenicity of more mature colon carcinoma cells, a phenomenon regulated by promoter methylation density [229]. Our protein expression analysis is in agreement with other studies showing that caveolins are down-regulated in differentiated Caco-2 cells [230], but contradicted by others, where caveolin-1 expression could be detected by western blotting [136].

Co-culture of Caco-2 cells with Raji B cells (a human hematopoietic cell line of B lymphocyte origin) induces an M-cell like phenotype in

**Table 2**  
Examples of principally different in vitro model systems of the intestinal barrier.

	Caco-2	HT-29	Caco-2 + Raji cells	Ussing
<i>Barrier component</i>				
Brush border peptidases	Reduced expression	Reduced expression	Reduced expression	Normal
Mucus	No secreted mucins	Secretes MUC2	No secreted mucins	Present, but mucin is discharged over time
Glycocalyx	Thin	Thin	Thin	Normal
Tight junctions	Very tight; low or no claudin 5 and 8	Tight	Tight; Low or no claudin 5 and 8	Normal
Endocytosis	Low activity Caveolins below LOQ	Low activity	Increased initial activity; detectable caveolins	Normal
Subepithelial tissue	Absent	Absent	Absent	Partly present; swells with time; lacks circulation, systemic immune system, and innervation
<i>Technical limitations</i>				
Viability	Extended	Extended	Extended	Limited to a few hours
Sensitivity to toxic compounds	High	High	High	Lower
Availability	Good	Limited	Limited	Exclusive
Equipment	Standard	Standard	Standard	Specialized
Technical challenge	Medium	Medium/High	High	High

LOQ: limit of quantitation.

Caco-2 cells [138,231]. Since the transformation to the FAE-like phenotype induces transcytotic capacity, the co-cultures have often been used to study transepithelial transport of antigens, pathogens, peptides, and nanoparticles. Nanoparticle transport could be increased further if the particles were decorated with an integrin-binding RGD-peptide [201]. Interestingly, the caveolin expression is increased in the M-cell like cells [230]. It is not clear to what extent this model represents the follicle associated epithelium. Since transcytosis in the model can be induced and stabilized by the addition of pro-inflammatory cytokines, it may also bear characteristics of the acutely inflamed intestine [232]. This is supported by studies in Caco-2 cell monolayers (without Raji B cells), which after a brief exposure to the enteropathogen *Yersinia pseudotuberculosis* increased the transport of nanoparticles 30 fold [233].

A triple culture model combining Caco-2, HT-29 and Raji B cells has been published [223]. As published previously for HT-29/Caco-2 co-cultures the triple culture model displayed a discontinuous mucus layer [222]. Polystyrene nanoparticle permeability was not studied in the triple model but cellular uptake of nanoparticles was determined to be 50% higher in the triple culture model than in pure Caco-2 culture. However, no statistics were given for this experiment so it is impossible to judge if this small difference is significant.

While Caco-2 cells is the most commonly used cell line for studying intestinal transport, several other cell lines have been employed in different applications (reviewed in [218]). T84 is a human colonic adenocarcinoma with a crypt-like phenotype [234]. The rat 2/4/A1 crypt cell-like cell line forms a leakier epithelium than the other cell lines described and has been used for transport and permeability of small molecule drugs [235,236]. Dog kidney epithelium derived Madine-Darby Canine Kidney (MDCK) cells are often used as a model system for transporter expression and as a model for epithelial transcytosis of macromolecules and nanoparticles [106].

#### 4.1. Novel advanced in vitro model systems

In recent years many new cell culture systems have been developed trying to improve cell phenotypes by mimicking key physiological parameters such as 3D-geometry, fluid flow and mechanical stimulation simulating pressure changes caused by the heartbeats.

For example human small intestinal epithelial cell monolayers differentiated from adult stem cells have recently been applied to prediction of human oral drug absorption [237]. Interestingly, the epithelial monolayers showed a more in vivo-like pore size distribution in the tight junctions than Caco-2 cell monolayers [238]. This implicates that they might be advantageous to use for permeability studies of low permeable compounds such as peptides, provided that they become generally available.

In another culture model, different intestinal cell lines were cultured at the air-liquid interface in semi-wet cultures with mechanical stimulation. Both polarization and mucus production were induced in the cell cultures [239].

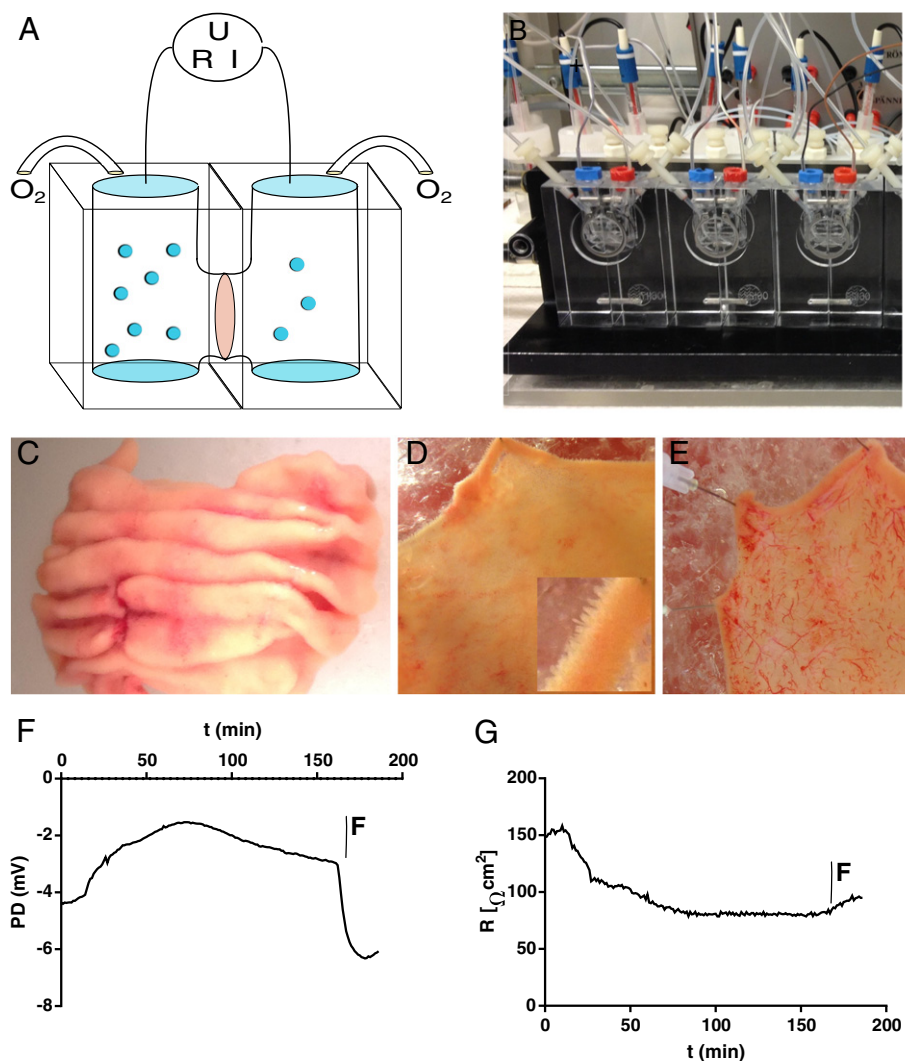
Caco-2 cells have also been cultured on micromolded collagen supports that mimicked villi morphology [240]. This led to increased expression of the transmembrane mucin MUC17 and the tight junction protein occludin [240]. MUC17 expression led to an increased protection against *S. typhimurium* infection.

The most advanced culture systems developed to date are the microfluidic organs-on-chips [241]. Gut-on-a-chip technology has also been used with Caco-2 cells [242,243]. The cells are cultured on a semi-permeable membrane with the apical and basolateral membranes facing two different fluid-filled channels simulating intestinal lumen and blood, respectively. Incorporating pressure pulses to simulate both heart beat and peristaltic movement leads to dramatic changes in the Caco-2 cell phenotype. Mucin expression was induced in the cultures and the Caco-2 cells started to form villous- and crypt-like structures [242,243]. Further differentiation of the Caco-2 cells into several intestinal cell types, including goblet cells, was observed [242].

In summary, more advanced, specialized cell culture models that may better mimic the in vivo state than the currently favored models reviewed here are under development. These novel models need confirmation and further study but could make significant contributions to the field in the future when they are well established. Still, it is difficult to capture the full complexity of the intestinal epithelium in these models (Table 2). Could studies of excised human intestinal tissues mounted in Ussing chambers provide a better model?

## 5. Ussing chambers

The Ussing chamber (Fig. 9) was devised in 1946 by the Danish physiologist Hans Henriksen Ussing for the study of water excretion through toad skin [244,245]. It has since been used for the study of transport properties, permeability and physiology of diverse epithelial tissues ranging from the gut to the retina. The Ussing chamber is one of the most advanced methods for ADME research in vitro and is used to investigate intestinal integrity and permeability. Very few papers have been published where Ussing chambers have been used to study the permeability of nanoparticles (24 in total as of February, 2016) and only 4 of these studies have used human intestinal tissues [137, 233,246,247]. A handful of studies have used Ussing chambers to study the permeability of peptides not formulated in nanoparticles, none of these have been performed using human tissues. Therefore, some of the examples below have been obtained from Ussing chamber studies with tissues from experimental animals, usually rodents.



**Fig. 9.** Ussing chambers. A. Schematic drawing of an Ussing chamber. The intestinal tissue sample is mounted in a window connecting two buffer-filled, oxygenated compartments. Peptide or particle permeation from the donor compartment (here on the left representing the mucosal, apical side) to the receiver compartment (here on the right representing the serosal, basolateral side) is monitored. The electrophysiological properties transepithelial electrical resistance (TEER), potential difference between the two chambers (resting potential; PD) and short-circuit current (Isc) of the tissue are used as indicators of tissue integrity and viability and are followed throughout the experiment. B. Photograph of an Ussing chamber corresponding to the one in A. C. A freshly isolated specimen of human jejunal mucosa before dissection. Note the prominent transversal folds in the presence of the submucosal muscle layer. D–E. Human jejunal mucosa after dissection which removes the subepithelial muscle layer. D. Mucosal view. Note the absence of transversal folds and the villi along the edge of the specimen. E. Serosal view. Note the capillary bed. F–G. Electrophysiology readouts during an Ussing experiment. F. Resting potential difference (PD) between the two chambers. Note the drop in PD, starting around 90 min. F indicates addition of the cAMP-agonist forskolin. The strong electrical response to forskolin indicates good viability of this tissue specimen. G. Transepithelial electrical resistance (TEER) across the epithelium. Note the drop in TEER during the initial phase of the experiment. This is due to slow warming of the tissue sample originally mounted at 4 °C and does not indicate a decrease in epithelial integrity.

Ussing chamber studies based on human tissue have traditionally used colon or ileum resected during cancer surgery. In recent years, the global obesity epidemic has given access to more proximal intestinal segments. This is because different bariatric surgery techniques are now among the most common procedures in surgery clinics in many parts of the world [248]. During Roux-en-Y gastric bypass small jejunum tissue samples can be donated without risk to the patient. This has led to an increase in the availability of human intestinal tissue for Ussing chamber experiments. Intestinal tissues from obese patients could possibly show somewhat higher permeability than intestines in average weight patients [249,250]. In rodents it is clear that obesity induced by a high fat diet can lead to a relatively mild inflammation in the intestine, sometimes but not always accompanied by a defect in intestinal barrier function [251]. The severity of the induced inflammation seems to be species and strain dependent [252,253]. The evidence for intestinal inflammation in obese humans is contradictory with several studies giving conflicting results with no clear indication of barrier defects [250]. Obese

patients with non-alcoholic steatosis have shown evidence of a mild barrier defect that was not observed in obese or lean patients. [254] While induced obesity in rodents is an acute phenomenon following an extreme diet, most obese humans have been overweight for many years prior to bariatric surgery. It is therefore likely that the intestinal inflammation in human obese patients is milder with smaller effects on tissue integrity.

It is of great importance that the laboratory performing the Ussing chamber experiments is situated in close proximity to the surgery or clinic as the tissues rapidly deteriorate after surgical resection. Transportation should occur under well-defined conditions and should take no longer than 30–60 min in order to avoid decreased tissue viability and integrity [255,256].

The Ussing chamber technique is not only logistically, but also technically challenging [255–257]. The intestinal epithelium from freshly excised tissue has to be dissected from the underlying tissues along the submucosa and mounted so that it covers a window between two

adjacent chambers (Fig. 9). The chambers are filled with a buffer containing nutrients needed by the tissue; this is commonly Krebs-Ringer, supplemented with amino acids, warmed to 37 °C, and bubbled with oxygen or carbogen. Ussing chambers are available both in vertical (Fig. 9) and horizontal (not shown) formats [202,257]. Horizontal Ussing chambers are essentially identical to Franz diffusion cells with the addition of electrophysiological monitoring. Franz diffusion cells are mainly used for skin permeability studies but have been used with intestinal tissues as well [202,258]. Peptides, nanoparticles or other materials of interest are added to the chamber on the apical (mucosal) side of the tissue and their passage over the epithelium and appearance in the basolateral (serosal) chamber is monitored. From these concentration-time data, the apparent permeability rate constant (Papp) can be calculated [217,255].

Care must be taken to ensure the continued viability and integrity of the tissue. As in cell monolayers, this is usually achieved by monitoring the permeability of hydrophilic markers (a low permeability is associated with intact tissue) and measuring the electrophysiological parameters of the tissue (Fig. 9). The commonly used electrophysiological read out trans-epithelial electrical resistance (TEER) is a comparatively blunt tool with which to monitor epithelial integrity, so the use of hydrophilic permeability markers is highly recommended [257]. A common technique to monitor tissue viability at the end of an experiment is to add the cAMP-agonist forskolin. If the tissue is still viable, the induced increase in cAMP will stimulate the current passing over the epithelium, mainly by activation of CFTR (Cystic fibrosis transmembrane conductance regulator, ABCC7) Cl<sup>-</sup> channels (Fig. 9) [259].

Cytotoxicity readouts such as ATP levels, lactate dehydrogenase (LDH) release and apoptosis detection can be used in Ussing chambers. Such assays are thought to give more physiologically relevant data on acute toxic effects than can be achieved in cell culture since the latter are often more sensitive to xenobiotics, pharmaceutical excipients and nanoparticle formulations [260,261,262]. The use of human tissue also presents other advantages, as many of the intestinal tissue functions are preserved (see Table 2). These responses are often species specific, especially in the case of peptide metabolism, inflammatory response and cytokine release after exposure to e.g. toxic nanoparticles. After a permeability measurement in an Ussing chamber, the tissue can be processed for microscopy and absorbed peptides or nanoparticles can be visualized by light, fluorescence, or electron microscopy.

It is important to note that, in several cases, ex vivo experiments with Ussing chambers are unable to recapture the in vivo situation. The viability of tissue in the chamber, even under conditions carefully mimicking physiological conditions, is typically limited to up to 3 h [255–257]. If maintained for longer times, gradual shedding of the loosely attached mucosa and emptying of goblet cells will increase.

Excised intestinal tissues are devoid of functional circulation and lymphatic drainage. This leads to water accumulation and concomitant tissue swelling in the Ussing chamber [257]. Water accumulation can be reduced by substituting glucose with mannitol in the buffer bathing the mucosal, apical, surface of the tissue. The absence of glucose shuts down sodium-dependent glucose transport into the epithelium and thus sodium and glucose accumulation in the serosal chamber; this reduces the buildup of an osmotic gradient and reduces swelling of the tissue [257]. It is likely that the lack of circulation can impede passage of especially larger molecules through the subepithelial layer and it is thus possible that the subepithelium presents more of a barrier in ex vivo experiments than it does in vivo. In addition, the tissue specimens lack innervation and the subepithelial muscle layer. Therefore, responses dependent on nerve signaling and peristaltic movements, respectively, will not be captured ex vivo. Further, as the tissue lacks circulation, toxicologic responses dependent on the systemic immune system cannot be studied.

In summary, correctly handled, freshly isolated human tissues mounted in Ussing chambers provide a sensitive system that provides more of the complexity of the human intestinal barrier in vivo than

cell culture models. A downside of this complexity is the technical challenge of the system, which limits its capacity. The Ussing chamber remains a low throughput tool and therefore, as much information as possible should be extracted from each experiment.

### 5.1. Translation from Ussing chamber experiments to in vivo intestinal absorption

An advantage of using human intestinal tissues in Ussing chambers is that it enables prediction of intestinal absorption in man for molecules with significant intestinal metabolism, such as peptides [255,263]. The translation of permeability experiments in Ussing chambers to in vivo intestinal absorption has therefore been established for small drug molecules in both rat and human [255,264]. When comparing the permeability of small tripeptides (Ile-Pro-Pro and Val-Pro-Pro) in Caco-2 monolayers, rat intestinal tissue ex vivo and perfused jejunal loops, it was found that Ussing chamber permeabilities were 5–10 fold higher than the corresponding values derived from Caco-2 measurements [196]. The permeabilities in the jejunal loops were not significantly different from measurements from the Ussing chambers. Further, the permeabilities in the jejunal loops were comparable to those observed in in vivo experiments. Interestingly, the more controlled conditions in the Ussing chamber experiments resulted in lower variability in permeability values than the in vivo experiments. The good in vitro–in vivo correlation suggested that the subepithelial tissue barrier in the Ussing chambers did not influence the permeability measurements of these small peptides to a significant extent under the applied conditions.

The number of studies performed with human tissues in Ussing chambers is too small to draw definitive conclusions about their applicability in studies of larger peptide drugs and nanoparticles. Some promising results have, however, been obtained. Ussing chambers have been used for prediction of peptide absorption for e.g. IgG monoclonal antibodies [265]. Hornby and coworkers investigated the expression of FcRn in different segments of the intestine from the duodenum to the distal colon in rat, cymologous monkeys and man. Segmental expression levels of FcRn were predictive of Ussing chamber IgG monoclonal antibody (mAb) permeability over tissues derived from different parts of the small intestine for human, rat and cymologous monkeys. Additionally, regional absorption of antibodies showed the same rank order after instillation in different intestinal segments of cymologous monkeys. In human tissue experiments, both FcRn expression and transepithelial transport of IgG mAb in Ussing chambers increased from the duodenum and distally towards the colon. Thus, in this case, the regional IgG mAb absorption could be predicted from Ussing chamber experiments and FcRn expression data. If the large size of mAbs is considered, it seems likely that Ussing chambers can be used to study the permeability of other large, soluble, peptides too.

A few studies of nanoparticle-mediated intestinal absorption have been performed in Ussing chambers, mainly using rodent tissue. Studies of paclitaxel loaded PEG-ylated cyclodextrin poly(anhydride) nanoparticles (where cyclodextrin-paclitaxel complexes were used to increase drug loading and the PEG-ylated surface aimed at mucodiffusivity) established a rank order correlation between permeabilities of different nanoparticle constructs and rat plasma AUC of paclitaxel after in vivo administration [266]. In another study, nanoparticles were derived from trimethyl chitosan (for permeation enhancement) and stabilized with poly  $\gamma$ -glutamic acid [267]. This backbone was conjugated to FOY-251, an active derivative of camostat mesylate, for peptidase inhibition purposes. The nanoparticles were decorated with a goblet cell targeting peptide, and loaded with insulin (mean final diameter of 250–300 nm). In this case, studies in Ussing chamber mounted rat jejunal tissues were used to predict the nanoparticle construct that would give the most efficient insulin-delivery in in vivo rat studies.

In a third study, calcitonin-loaded solid lipid nanoparticles with densely PEG-ylated surfaces were decorated with a goblet cell targeting peptide or a CPP (mean diameters of 240–400 nm) [268]. Ussing

chamber experiments with rat duodenum could here predict the rank order and fold increase in *in vivo* rat bioavailability when comparing free calcitonin, solid lipid nanoparticles, goblet cell targeting peptide decorated solid lipid nanoparticles, and CPP-decorated solid lipid nanoparticles.

In a fourth study, Ussing chamber experiments were performed using human colonic tissues from inflammatory bowel disease (IBD) patients. PLGA nanoparticles (250 nm) and microparticles (3  $\mu\text{m}$ ) showed very limited permeability (measured in the receiving chamber of the Ussing chamber) in normal epithelium, while they showed increased permeability in the corresponding inflamed epithelium [246]. Penetration was measured as the number of particles found in microscopy sections of tissue specimens after exposure in Ussing chambers. Penetration, like permeability, increased significantly in the inflamed epithelium. Similarly, after *in vivo* administration of nano- and microparticles to the colon of IBD patients, only a few nanoparticles could be seen in the mucosa of patients with ulcerative colitis in remission. A larger accumulation of nanoparticles could be seen at active inflamed sites. Very few microparticles could be detected in the mucosa of Crohn's disease (CD) patients with mild inflammation. In rectal lesions in CD patients with severe inflammation a pronounced accumulation of microparticles could be seen, correlating well with the results from the Ussing chambers.

Tissue penetration and permeability also increased in the normal human ileum after a brief exposure to the enteropathogenic bacterium *Y. pseudotuberculosis*, presumably as a result of an acute inflammatory response [233]. The enhanced transepithelial transport was inhibited by the commonly used but unselective endocytosis inhibitor 5-(N-ethyl-N-isopropyl) amiloride (EIPA), indicating the involvement of an endocytotic mechanism, possibly macropinocytosis [141,233].

PEG-functionalized, chitosan-functionalized, and non-functionalized PLGA nano- and microparticles were also used in Ussing chamber experiments with colon tissue biopsies derived from patients with IBD. It could be shown that PEG-ylation increased tissue permeation and penetration in Ussing chamber experiments [247]. In this study, neither nano- nor micro-PLGA particles (250 nm and 3  $\mu\text{m}$ , respectively) showed any permeability over colonic epithelium. Healthy and inflamed colons were both impermeable to the PLGA nanoparticles. Both PEG-ylated nano- and microparticles showed permeability over the inflamed mucosa and, to a small extent over the healthy epithelium. Nanoparticles were more permeable than the microparticles in both tissue types. The same pattern was found in microscopy analysis of tissue samples from the Ussing chambers. Inflamed mucosa contained many more particles (both micro and nano) than healthy tissue. In all cases, nanoparticles were detected in higher numbers in the tissue than microparticles. The data suggests that the PEG-ylated particles had mucus penetrating properties because of their short, 5 kDa PEG-chains, as discussed in Section 3.2.2 and therefore they could penetrate the mucus layer to interact with the epithelium. Chitosan-functionalized positively charged

nano- and microparticles showed a pronounced binding to mucus and cell surfaces preventing the permeability and uptake of these particles.

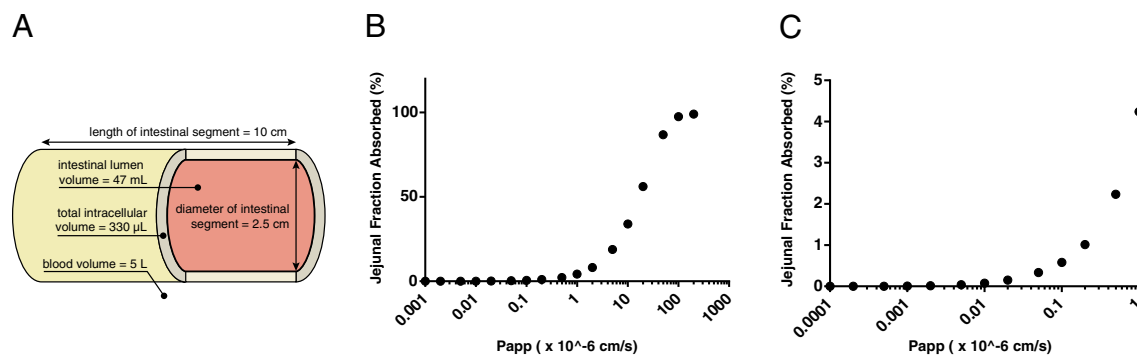
In summary, these results show a good *in vitro*-*in vivo* agreement between nanoparticle uptake in normal as well as inflamed human intestinal tissue. They also show that the uptake of non-targeted nanoparticles increases, but remains low, in IBD.

## 6. Modeling of jejunal peptide or nanoparticle absorption

In a recent study, we introduced a simple mechanistic model for prediction of the intestinal regional fraction absorbed from permeability (Papp) values derived from Caco-2 or Ussing chamber experiments [14]. The model was set up to mimic a typical human intestinal perfusion experiment in a 10-cm intestinal segment (loc-i-gut) [96, 269]. The model assumes a transit time of 270 min, that the permeant is in solution and stable, evenly distributed in the lumen during the study, and that the transport rates are in the linear range. Based on these assumptions the fraction absorbed from the perfusate can be predicted. Comparing the model to previously published Ussing permeability studies revealed a good correspondence to human *in vivo* fraction absorbed values [255].

The geometry of the intestinal segment modeled is illustrated in Fig. 10A. Fig. 10B shows the predicted fraction absorbed as a function of intestinal permeability. The intestinal fraction absorbed increases from 1% to 99.5% when the permeability coefficient (Papp) increases from 0.5 to  $100 \times 10^{-6}$  cm/s. Most peptides and nanoparticles show low Papp values that are predicted to correspond to low (far below 1%) absorbed fractions *in vivo*. Exceptions to this general statement exist, notably, the insulin permeation studies conducted after MLCP inhibition by Taverner and coworkers [66] as well as the utilization of targeted nanoparticles such as the IgG Fc-decorated nanoparticles used to achieve FcRn mediated nanoparticle transcytosis performed by Pridgen et al., [109].

In the study of Taverner et al., an insulin bioavailability of 3–4% after tight junction modulation was calculated, corresponding to a Papp value of  $0.8\text{--}0.9 \times 10^{-6}$  cm/s [66]. In the study of Pridgen et al., the bioavailability was not calculated for the Fc-decorated nanoparticles [109]. However, considering that the dose of 1.1 IU/kg insulin corresponds to a typical daily subcutaneous dose in man, and the resulting decrease in blood glucose was 45%, it is likely that the bioavailability >25%, corresponding to a Papp value  $>5 \times 10^{-6}$  cm/s. Targeting of peptide or nanoparticles to the vitamin B12 absorption pathway resulted in bioavailabilities estimated to be in the range of 5–25% [113,115,116,180]. This corresponds to Papp values in the range of  $1\text{--}5 \times 10^{-6}$  cm/s. A bias inherent in our simple predictions is that the model assumes exposure to a constant concentration of the respective delivery system. *In vivo*, an added complexity is that the concentration will vary over time. Finally, it is note-worthy that these studies were performed in small



**Fig. 10.** Prediction of intestinal regional fraction absorbed from Papp values derived from Ussing chamber experiments. Predictions were made using the model described in Ölander et al. [14]. A. shows a schematic drawing of the model. B. Predicted fraction absorbed as a function of permeability coefficient (Papp) C. An enlargement of the graph for the lower Papp values typical of nanoparticles is shown.



animal species and may not be easily translated to larger species, including man.

## 7. Summary and conclusions

There are significant physiological and biochemical barriers to peptide absorption in the gastrointestinal tract. Nanoparticles have often been seen as a tool to overcome these barriers. Our review shows that to be effective, such nanoparticles must possess certain characteristics. First they must be able to protect their cargo against enzymatic breakdown in the gut lumen and by intestinal cells. Second, they should possess characteristics that allow them to pass or deliver the peptide across the mucus barrier. Finally, they should pass the intestinal epithelium lining the intestinal lumen. To circumvent this problem, many oral peptide delivery systems in current clinical trials have incorporated absorption enhancers with more or less non-specific mechanisms of action in their formulations. Here, we have reviewed alternative approaches and identified complex but more specific principles for oral peptide delivery, in particular in nanoparticles.

To pass the epithelial layer two strategies have been used with some success: the paracellular route via the tight junctions and the transcellular transcytosis route. Tight junctions can be modulated by MLC phosphorylation via MLCP inhibition, resulting in significant absorption of peptides. The transcellular pathways via comparably well investigated transcytotic pathways such as those for vitamin B12 and IgG were identified as the most promising options for the oral delivery of peptides encapsulated in nanoparticles. Importantly, to become a viable alternative for oral peptide delivery to humans, the promising results observed with tight junction regulation and transcytosis in small animals must be translatable to man.

## Acknowledgment

This work was supported by The European Union Seventh Framework Programme Trans-Int (grant agreement number NMP4-LA-2012-281035) and the Swedish Research Council (no. 8222). We thank our clinical collaborators, Professor Magnus Sundbom (Department of Surgical Sciences, Upper Abdominal Surgery, Uppsala University) and coworkers, and Professor Mikael Wirén (Vrinnevi Hospital, Norrköping and Department of Clinical and Experimental Medicine, Oncology, Linköping University) and coworkers for their excellent help with tissue collection. The help with Ussing chamber experiments by the group of Professor Johan D Söderholm and Åsa Keita (Department of Clinical and Experimental Medicine, Division of Surgery, Linköping University) is gratefully acknowledged. The authors are indebted to Ivailo Simoff, Georgiy Khodus and Magnus Ölander, Department of Pharmacy, Uppsala University for their help in many aspects of this work.

## References

- 1] A.A. Date, J. Hanes, L.M. Ensign, Nanoparticles for oral delivery: design, evaluation and state-of-the-art, *J. Control. Release* (2016).
- 2] H. Sinnecker, T. Krause, S. Koelling, I. Lautenschläger, A. Frey, The gut wall provides an effective barrier against nanoparticle uptake, *Beilstein J. Nanotechnol.* 5 (2014) 2092–2101.
- 3] X. Hu, W. Fan, Z. Yu, Y. Lu, J. Qi, J. Zhang, X. Dong, W. Zhao, W. Wu, Evidence does not support absorption of intact solid lipid nanoparticles via oral delivery, *Nanoscale* 8 (2016) 7024–7035.
- 4] N. Tsomaia, Peptide therapeutics: targeting the undruggable space, *Eur. J. Med. Chem.* 94 (2015) 459–470.
- 5] B.C. Doak, B. Over, F. Giordanetto, J. Kihlberg, Oral druggable space beyond the rule of 5: insights from drugs and clinical candidates, *Chem. Biol.* 21 (2014) 1115–1142.
- 6] K. Palm, P. Stenberg, K. Luthman, P. Artursson, Polar molecular surface properties predict the intestinal absorption of drugs in humans, *Pharm. Res.* 14 (1997) 568–571.
- 7] *Physiology of the Gastrointestinal Tract*, Raven Press, 2006.
- 8] P.R. Kiela, F.K. Ghishan, Physiology of intestinal absorption and secretion, *Best Pract. Res. Clin. Gastroenterol.* 30 (2016) 145–159.
- 9] B.E. Goodman, Insights into digestion and absorption of major nutrients in humans, *Adv. Physiol. Educ.* 34 (2010) 44–53.
- 10] H.J. Binder, A. Reuben, Nutrient digestion and absorption, in: W.F. Boron, E.L. Boulpaep (Eds.), *Medical Physiology*, Elsevier, Philadelphia, PA, USA 2012, pp. 949–979.
- 11] D.C. Whitcomb, M.E. Lowe, Human pancreatic digestive enzymes, *Dig. Dis. Sci.* 52 (2007) 1–17.
- 12] M. Uhlen, L. Fagerberg, B.M. Hallström, C. Lindskog, P. Oksvold, A. Mardinoglu, Å. Sivertsson, C. Kampf, E. Sjöstedt, A. Asplund, Tissue-based map of the human proteome, *Science* 347 (2015) 1260419.
- 13] M. Uhlen, P. Oksvold, L. Fagerberg, E. Lundberg, K. Jonasson, M. Forsberg, M. Zwahlen, C. Kampf, K. Wester, S. Hober, Towards a knowledge-based human protein atlas, *Nat. Biotechnol.* 28 (2010) 1248–1250.
- 14] M. Ölander, J.R. Wiśniewski, P. Matsson, P. Lundquist, P. Artursson, The proteome of filter-grown Caco-2 cells with a focus on proteins involved in drug disposition, *J. Pharm. Sci.* (2016).
- 15] I. Hubatsch, L. Lazorova, A. Vahlne, P. Artursson, Orally active antiviral tripeptide glycyl-prolyl-glycinamide is activated by CD26 (dipeptidyl peptidase IV) before transport across the intestinal epithelium, *Antimicrob. Agents Chemother.* 49 (2005) 1087–1092.
- 16] A. Bak, O.S. Gudmundsson, G.J. Friis, T.J. Siahaan, R.T. Borchardt, Acyloxyalkoxy-based cyclic prodrugs of opioid peptides: evaluation of the chemical and enzymatic stability as well as their transport properties across Caco-2 cell monolayers, *Pharm. Res.* 16 (1999) 24–29.
- 17] A.L. Ungell, A. Andreasson, K. Lundin, L. Utter, Effects of enzymatic inhibition and increased paracellular shunting on transport of vasopressin analogues in the rat, *J. Pharm. Sci.* 81 (1992) 640–645.
- 18] M. Koziolok, M. Grimm, F. Schneider, P. Jedamzik, M. Sager, J.-P. Kühn, W. Siegmund, W. Weitschies, Navigating the human gastrointestinal tract for oral drug delivery: uncharted waters and new frontiers, *Adv. Drug Deliv. Rev.* 101 (2016) 75–88.
- 19] S. Davis, J. Hardy, J. Fara, Transit of pharmaceutical dosage forms through the small intestine, *Gut* 27 (1986) 886–892.
- 20] H.C. Lin, C. Prather, R.S. Fisher, J.H. Meyer, R.W. Summers, M. Pimentel, R.W. McCallum, L.M. Akkermans, V. Loening-Baucke, A.T.F.Co.G. Transit, Measurement of gastrointestinal transit, *Dig. Dis. Sci.* 50 (2005) 989–1004.
- 21] T.T. Kararli, Comparison of the gastrointestinal anatomy, physiology, and biochemistry of humans and commonly used laboratory animals, *Biopharm. Drug Dispos.* 16 (1995) 351–380.
- 22] T. Takashima, T. Shingaki, Y. Katayama, E. Hayashinaka, Y. Wada, M. Kataoka, D. Ozaki, H. Doi, M. Suzuki, S. Ishida, Dynamic analysis of fluid distribution in the gastrointestinal tract in rats: positron emission tomography imaging after oral administration of nonabsorbable marker, [18F] deoxyfluoropoly (ethylene glycol), *Mol. Pharm.* 10 (2013) 2261–2269.
- 23] H. Lennernäs, C.-G. Regårdh, Regional gastrointestinal absorption of the beta-blocker pafenolol in the rat and intestinal transit rate determined by movement of 14C-polyethylene glycol (PEG) 4000, *Pharm. Res.* 10 (1993) 130–135.
- 24] C. Picard, J. Wysocki, J. Fioramonti, N. Griffiths, Intestinal and colonic motor alterations associated with irradiation-induced diarrhoea in rats, *Neurogastroenterol. Motil.* 13 (2001) 19–26.
- 25] T. Aguirre, D. Teijeiro-Osorio, M. Rosa, I. Coulter, M. Alonso, D. Brayden, Current status of selected oral peptide technologies in advanced preclinical development and in clinical trials, *Adv. Drug Deliv. Rev.* 106 (2016) 223–241.
- 26] T.A. Aguirre, M. Rosa, I.S. Coulter, D.J. Brayden, In vitro and in vivo preclinical evaluation of a minisphere emulsion-based formulation (SmPill®) of salmon calcitonin, *Eur. J. Pharm. Sci.* 79 (2015) 102–111.
- 27] I. Toth, J.P. Malkinson, N.S. Flinn, B. Drouillard, A. Horvath, J. Erchegeyi, M. Idei, A. Venetianer, P. Artursson, L. Lazorova, B. Szende, G. Keri, Novel liposomal acid- and liposaccharide-based system for peptide delivery: application for oral administration of tumor-selective somatostatin analogues, *J. Med. Chem.* 42 (1999) 4010–4013.
- 28] T. Pelaseyed, J.H. Bergström, J.K. Gustafsson, A. Ermund, G.M. Birchenough, A. Schütte, S. Post, F. Svensson, A.M. Rodríguez-Piñeiro, E.E. Nyström, The mucus and mucins of the goblet cells and enterocytes provide the first defense line of the gastrointestinal tract and interact with the immune system, *Immunol. Rev.* 260 (2014) 8–20.
- 29] M.E. Johansson, D. Ambort, T. Pelaseyed, A. Schütte, J.K. Gustafsson, A. Ermund, D.B. Subramani, J.M. Holmén-Larsson, K.A. Thomsson, J.H. Bergström, Composition and functional role of the mucus layers in the intestine, *Cell. Mol. Life Sci.* 68 (2011) 3635–3641.
- 30] A.P. Corfield, Mucins: a biologically relevant glycan barrier in mucosal protection, *Biochim. Biophys. Acta Gen. Subj.* 1850 (2015) 236–252.
- 31] N. Jonckheere, N. Skrypek, F. Frénois, I. Van Seuningen, Membrane-bound mucin modular domains: from structure to function, *Biochimie* 95 (2013) 1077–1086.
- 32] J.R. Wiśniewski, P. Ostasiewicz, K. Duś, D.F. Zielińska, F. Gnad, M. Mann, Extensive quantitative remodeling of the proteome between normal colon tissue and adenocarcinoma, *Mol. Syst. Biol.* 8 (2012).
- 33] M.E. Johansson, G.C. Hansson, Mucus and the goblet cell, *Dig. Dis. Sci.* 31 (2013) 305–309.
- 34] D. Ambort, M.E. Johansson, J.K. Gustafsson, H.E. Nilsson, A. Ermund, B.R. Johansson, P.J. Koek, H. Hebert, G.C. Hansson, Calcium and pH-dependent packing and release of the gel-forming MUC2 mucin, *Proc. Natl. Acad. Sci.* 109 (2012) 5645–5650.
- 35] L.A. van der Waaij, H.J. Harmsen, M. Madjipour, F.G. Kroese, M. Zwiers, H. Van Dulleman, N. De Boer, G. Welling, P.L. Jansen, Bacterial population analysis of human colon and terminal ileum biopsies with 16S rRNA-based fluorescent probes: commensal bacteria live in suspension and have no direct contact with epithelial cells, *Inflamm. Bowel Dis.* 11 (2005) 865–871.

- [36] S.K. Lai, Y.-Y. Wang, J. Hanes, Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tissues, *Adv. Drug Deliv. Rev.* 61 (2009) 158–171.
- [37] C. Atuma, V. Strugala, A. Allen, L. Holm, The adherent gastrointestinal mucus gel layer: thickness and physical state in vivo, *Am. J. Physiol. Gastrointest. Liver Physiol.* 280 (2001) G922–G929.
- [38] G.C. Hansson, M.E. Johansson, The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria, *Gut Microbes* 1 (2010) 51–54.
- [39] B.H. Bajka, N.M. Rigby, K.L. Cross, A. Macierzanka, A.R. Mackie, The influence of small intestinal mucus structure on particle transport ex vivo, *Colloids Surf. B: Biointerfaces* 135 (2015) 73–80.
- [40] Z. Hong, B. Chasan, R. Bansil, B.S. Turner, K.R. Bhaskar, N.H. Afdhal, Atomic force microscopy reveals aggregation of gastric mucin at low pH, *Biomacromolecules* 6 (2005) 3458–3466.
- [41] J.R. Gum, J. Byrd, J.W. Hicks, N. Toribara, D. Lampert, Y. Kim, Molecular cloning of human intestinal mucin cDNAs. Sequence analysis and evidence for genetic polymorphism, *J. Biol. Chem.* 264 (1989) 6480–6487.
- [42] M. Rong, E.A. Rossi, J. Zhang, R.R. McNeer, J.M. van den Brande, M. Yasin, D.T. Weed, C.A. Carothers Carraway, J.F. Thompson, K.L. Carraway, Expression and localization of Muc4/sialomucin complex (SMC) in the adult and developing rat intestine: implications for Muc4/SMC function, *J. Cell. Physiol.* 202 (2005) 275–284.
- [43] M.R. Neutra, N.J. Mantis, A. Frey, P.J. Giannasca, The composition and function of M cell apical membranes: implications for microbial pathogenesis, *Semin. Immunol.* 11 (1999) 171–181.
- [44] A. Frey, K.T. Giannasca, R. Weltzin, P.J. Giannasca, H. Reggio, W.I. Lencer, M.R. Neutra, Role of the glycocalyx in regulating access of microparticles to apical plasma membranes of intestinal epithelial cells: implications for microbial attachment and oral vaccine targeting, *J. Exp. Med.* 184 (1996) 1045–1059.
- [45] I. Chantret, A. Barbat, E. Dussaulx, M.G. Brattain, A. Zweibaum, Epithelial polarity, villin expression, and enterocytic differentiation of cultured human colon carcinoma cells: a survey of twenty cell lines, *Cancer Res.* 48 (1988) 1936–1942.
- [46] T.E. Phillips, C. Huet, P.R. Bilbo, D.K. Podolsky, D. Louvard, M.R. Neutra, Human intestinal goblet cells in monolayer culture: characterization of a mucus-secreting subclone derived from the HT29 colon adenocarcinoma cell line, *Gastroenterology* 94 (1988) 1390–1403.
- [47] G. Birchenough, M.E. Johansson, J. Gustafsson, J. Bergström, G. Hansson, New developments in goblet cell mucus secretion and function, *Mucosal Immunol.* 8 (2015) 712–719.
- [48] M.A. Vieira, T.A. Gomes, A.J. Ferreira, T. Knöbl, A.L. Servin, V. Liévin-Le Moal, Two atypical enteropathogenic *Escherichia coli* strains induce the production of secreted and membrane-bound mucins to restrict their own growth at the apical surface of human mucin-secreting intestinal HT29-MTX cells, *Infect. Immun.* 78 (2010) 927–938.
- [49] P.C. Griffiths, B. Cattoz, M.S. Ibrahim, J.C. Anuonye, Probing the interaction of nanoparticles with mucin for drug delivery applications using dynamic light scattering, *Eur. J. Pharm. Biopharm.* 97 (2015) 218–222.
- [50] O. Lieliegl, I. Vladescu, K. Ribbeck, Characterization of particle translocation through mucin hydrogels, *Biophys. J.* 98 (2010) 1782–1789.
- [51] J.S. Fordtran, T.W. Locklear, Ionic constituents and osmolality of gastric and small-intestinal fluids after eating, *Am. J. Dig. Dis.* 11 (1966) 503–521.
- [52] H.M. Yildiz, L. Speciner, C. Ozdemir, D.E. Cohen, R.L. Carrier, Food-associated stimuli enhance barrier properties of gastrointestinal mucus, *Biomaterials* 54 (2015) 1–8.
- [53] S.S. Datta, A.P. Steinberg, R.F. Ismagilov, Polymers in the gut compress the colonic mucus hydrogel, *Proc. Natl. Acad. Sci.* (2016) 201602789.
- [54] S.S. Olmsted, J.L. Padgett, A.I. Yudin, K.J. Whaley, T.R. Moench, R.A. Cone, Diffusion of macromolecules and virus-like particles in human cervical mucus, *Biophys. J.* 81 (2001) 1930–1937.
- [55] S.K. Lai, Y.-Y. Wang, K. Hida, R. Cone, J. Hanes, Nanoparticles reveal that human cervicovaginal mucus is riddled with pores larger than viruses, *Proc. Natl. Acad. Sci. U.S.A.* (2010) 598–603.
- [56] Q. Xu, L.M. Ensign, N.J. Boylan, A. Schön, X. Gong, J.-C. Yang, N.W. Lamb, S. Cai, T. Yu, E. Freire, Impact of surface polyethylene glycol (PEG) density on biodegradable nanoparticle transport in mucus ex vivo and distribution in vivo, *ACS Nano* 9 (2015) 9217–9227.
- [57] L.M. Ensign, B.C. Tang, Y.-Y. Wang, A.T. Terence, T. Hoen, R. Cone, J. Hanes, Mucus-penetrating nanoparticles for vaginal drug delivery protect against herpes simplex virus, *Sci. Transl. Med.* 4 (2012) 138ra179.
- [58] J.C. Swavola, T.D. Edwards, M.A. Bevan, Direct measurement of macromolecule-coated colloid–mucus interactions, *Langmuir* 31 (2015) 9076–9085.
- [59] W. Shan, X. Zhu, M. Liu, L. Li, J. Zhong, W. Sun, Z. Zhang, Y. Huang, Overcoming the diffusion barrier of mucus and absorption barrier of epithelium by self-assembled nanoparticles for oral delivery of insulin, *ACS Nano* 9 (2015) 2345–2356.
- [60] P. Artursson, S.D. Knight, Breaking the intestinal barrier to deliver drugs, *Science* 347 (2015) 716–717.
- [61] J.R. Turner, M.M. Buschmann, I. Romero-Calvo, A. Sailer, L. Shen, The role of molecular remodeling in differential regulation of tight junction permeability, *Semin. Cell Dev. Biol.* 36 (2014) 204–212.
- [62] C. Barmeyer, J.D. Schulzke, M. Fromm, Claudin-related intestinal diseases, *Semin. Cell Dev. Biol.* 42 (2015) 30–38.
- [63] D. Günzel, S. Alan, Claudins and the modulation of tight junction permeability, *Physiol. Rev.* 93 (2013) 525–569.
- [64] R.J. Mrsny, G.T. Brown, K. Gerner-Smidt, A.G. Buret, J.B. Meddings, C. Quan, M. Koval, A. Nusrat, A key claudin extracellular loop domain is critical for epithelial barrier integrity, *Am. J. Pathol.* 172 (2008) 905–915.
- [65] H. Suzuki, T. Nishizawa, K. Tani, Y. Yamazaki, A. Tamura, R. Ishitani, N. Dohmae, S. Tsukita, O. Nureki, Y. Fujiyoshi, Crystal structure of a claudin provides insight into the architecture of tight junctions, *Science* 344 (2014) 304–307.
- [66] A. Tavernier, R. Dondi, K. Almansour, F. Laurent, S.-E. Owens, I.M. Eggleston, N. Fotaki, R.J. MRSny, Enhanced paracellular transport of insulin can be achieved via transient induction of myosin light chain phosphorylation, *J. Control. Release* 210 (2015) 189–197.
- [67] M. Eto, Regulation of cellular protein phosphatase-1 (PP1) by phosphorylation of the CPI-17 family, C-kinase-activated PP1 inhibitors, *J. Biol. Chem.* 284 (2009) 35273–35277.
- [68] A.L. Lameris, S. Huybers, K. Kaukinen, T.H. Mäkelä, R.J. Bindels, J.G. Hoenderop, P.I. Nevalainen, Expression profiling of claudins in the human gastrointestinal tract in health and during inflammatory bowel disease, *Scand. J. Gastroenterol.* 48 (2013) 58–69.
- [69] A. Tamura, H. Hayashi, M. Imasato, Y. Yamazaki, A. Hagiwara, M. Wada, T. Noda, M. Watanabe, Y. Suzuki, S. Tsukita, Loss of claudin-15, but not claudin-2, causes Na<sup>+</sup> deficiency and glucose malabsorption in mouse small intestine, *Gastroenterology* 140 (2011) 913–923.
- [70] S. Noda, S. Tanabe, T. Suzuki, Naringenin enhances intestinal barrier function through the expression and cytoskeletal association of tight junction proteins in Caco-2 cells, *Mol. Nutr. Food Res.* 57 (2013) 2019–2028.
- [71] C.R. Weber, D.R. Raleigh, L. Su, L. Shen, E.A. Sullivan, Y. Wang, J.R. Turner, Epithelial myosin light chain kinase activation induces mucosal interleukin-13 expression to alter tight junction ion selectivity, *J. Biol. Chem.* 285 (2010) 12037–12046.
- [72] D.R. Clayburgh, T.A. Barrett, Y. Tang, J.B. Meddings, L.J. Van Eldik, D.M. Watterson, L.L. Clarke, R.J. MRSny, J.R. Turner, Epithelial myosin light chain kinase-dependent barrier dysfunction mediates T cell activation-induced diarrhea in vivo, *J. Clin. Invest.* 115 (2005) 2702–2715.
- [73] Y. Zolotarevsky, G. Hecht, A. Koutsouris, D.E. Gonzalez, C. Quan, J. Tom, R.J. MRSny, J.R. Turner, A membrane-permeant peptide that inhibits MLC kinase restores barrier function in vitro models of intestinal disease, *Gastroenterology* 123 (2002) 163–172.
- [74] F. Wang, W.V. Graham, Y. Wang, E.D. Witkowski, B.T. Schwarz, J.R. Turner, Interferon-gamma; and Tumor Necrosis Factor-alpha; Synergize to Induce Intestinal Epithelial Barrier Dysfunction by Up-Regulating Myosin Light Chain Kinase Expression, *Am. J. Pathol.* 166 (2005) 409–419.
- [75] D.R. Clayburgh, M.W. Musch, M. Leitges, Y.-X. Fu, J.R. Turner, Coordinated epithelial NHE3 inhibition and barrier dysfunction are required for TNF-mediated diarrhea in vivo, *J. Clin. Invest.* 116 (2006) 2682–2694.
- [76] A. Casselbrant, E. Elias, L. Fändriks, V. Wallenius, Expression of tight-junction proteins in human proximal small intestinal mucosa before and after Roux-en-Y gastric bypass surgery, *Surg. Obes. Relat. Dis.* 11 (2015) 45–53.
- [77] S. Zeissig, N. Bürgel, D. Günzel, J. Richter, J. Mankertz, U. Wahnschaffe, A.J. Kroesen, M. Zeitz, M. Fromm, J.D. Schulzke, Changes in expression and distribution of claudin 2, 5 and 8 lead to discontinuous tight junctions and barrier dysfunction in active Crohn's disease, *Gut* 56 (2007) 61–72.
- [78] N. Bürgel, C. Bojarski, J. Mankertz, M. Zeitz, M. Fromm, J.D. Schulzke, Mechanisms of diarrhea in collagenous colitis, *Gastroenterology* 123 (2002) 433–443.
- [79] M. Campbell, F. Hanrahan, O.L. Gobbo, M.E. Kelly, A.-S. Kiang, M.M. Humphries, A.T. Nguyen, E. Ozaki, J. Keaney, C.W. Blau, Targeted suppression of claudin-5 decreases cerebral oedema and improves cognitive outcome following traumatic brain injury, *Nat. Commun.* 3 (2012) 849.
- [80] T. Lindmark, T. Nikkilä, P. Artursson, Mechanisms of absorption enhancement by medium chain fatty acids in intestinal epithelial Caco-2 cell monolayers, *J. Pharmacol. Exp. Ther.* 275 (1995) 958–964.
- [81] S. Maher, R. Kennelly, V.A. Bzik, A.W. Baird, X. Wang, D. Winter, D.J. Brayden, Evaluation of intestinal absorption enhancement and local mucosal toxicity of two promoters. I. Studies in isolated rat and human colonic mucosae, *Eur. J. Pharm. Sci.* 38 (2009) 291–300.
- [82] T. Lindmark, J.D. Söderholm, G. Olaison, G. Alván, G. Ocklind, P. Artursson, Mechanism of absorption enhancement in humans after rectal administration of ampicillin in suppositories containing sodium caprate, *Pharm. Res.* 14 (1997) 930–935.
- [83] M. Terrak, F. Kerff, K. Langsetmo, T. Tao, R. Dominguez, Structural basis of protein phosphatase 1 regulation, *Nature* 429 (2004) 780–784.
- [84] J.J. Berglund, M. Riegler, Y. Zolotarevsky, E. Wenzl, J.R. Turner, Regulation of human jejunal transmucosal resistance and MLC phosphorylation by Na<sup>+</sup>-glucose co-transport, *Am. J. Physiol. Gastrointest. Liver Physiol.* 281 (2001) G1487–G1493.
- [85] L.M. Feighery, S.W. Cochrane, T. Quinn, A.W. Baird, D. O'Toole, S.-E. Owens, D. O'Donoghue, R.J. MRSny, D.J. Brayden, Myosin light chain kinase inhibition: correction of increased intestinal epithelial permeability in vitro, *Pharm. Res.* 25 (2008) 1377–1386.
- [86] A.C. Kemper, R.D. Specian, Rat small intestinal mucins: a quantitative analysis, *Anat. Rec.* 229 (1991) 219–226.
- [87] T.D. Grant, R.D. Specian, Proliferation of goblet cells and vacuolated cells in the rabbit distal colon, *Anat. Rec.* 252 (1998) 41–48.
- [88] O. Mace, B. Tehan, F. Marshall, Pharmacology and physiology of gastrointestinal enteroendocrine cells, *Pharmacol. Res. Perspect.* 3 (2015).
- [89] S.M. Karam, Lineage commitment and maturation of epithelial cells in the gut, *Front. Biosci.* 4 (1999) D286–D298.
- [90] A.M. Mowat, W.W. Agace, Regional specialization within the intestinal immune system, *Nat. Rev. Immunol.* 14 (2014) 667–685.
- [91] J. Söderholm, The intestinal barrier and its regulation by neuroimmune factors, *Neurogastroenterol. Motil.* 22 (2010) 718–733.
- [92] R.L. Owen, Sequential uptake of horseradish peroxidase by lymphoid follicle epithelium of Peyer's patches in the normal unobstructed mouse intestine: an ultrastructural study, *Gastroenterology* 72 (1977) 440–451.
- [93] M.R. Neutra, A. Frey, J.-P. Kraehenbuhl, Epithelial M cells: gateways for mucosal infection and immunization, *Cell* 86 (1996) 345–348.
- [94] A. Ermund, J.K. Gustafsson, G.C. Hansson, Å.V. Keita, Mucus properties and goblet cell quantification in mouse, rat and human ileal Peyer's patches, *PLoS One* 8 (2013), e83688.

- [95] J.H. Niess, H.C. Reinecker, Dendritic cells in the recognition of intestinal microbiota, *Cell. Microbiol.* 8 (2006) 558–564.
- [96] H.F. Helander, L. Fändriks, Surface area of the digestive tract—revisited, *Scand. J. Gastroenterol.* 49 (2014) 681–689.
- [97] W.M. Miner-Williams, B.R. Stevens, P.J. Moughan, Are intact peptides absorbed from the healthy gut in the adult human? *Nutr. Res. Rev.* 27 (2014) 308–329.
- [98] D.E. Smith, B. Cléménçon, M.A. Hediger, Proton-coupled oligopeptide transporter family SLC15: physiological, pharmacological and pathological implications, *Mol. Asp. Med.* 34 (2013) 323–336.
- [99] E. Vives, P. Brodin, B. Lebleu, A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus, *J. Biol. Chem.* 272 (1997) 16010–16017.
- [100] W.B. Kauffman, T. Fuselier, J. He, W.C. Wimley, Mechanism matters: a taxonomy of cell penetrating peptides, *Trends Biochem. Sci.* 40 (2015) 749–764.
- [101] E. Dupont, A. Prochiantz, A. Joliot, Penetratin story: an overview, *Cell-Penetrating Pept. Methods Protoc.* (2015) 29–37.
- [102] J.R. Marks, J. Placone, K. Hristova, W.C. Wimley, Spontaneous membrane-translocating peptides by orthogonal high-throughput screening, *J. Am. Chem. Soc.* 133 (2011) 8995–9004.
- [103] M. Kristensen, H.M. Nielsen, Cell-penetrating peptides as carriers for oral delivery of biopharmaceuticals, *Basic Clin. Pharmacol. Toxicol.* 118 (2016) 99–106.
- [104] S. Zhu, S. Chen, Y. Gao, F. Guo, F. Li, B. Xie, J. Zhou, H. Zhong, Enhanced oral bioavailability of insulin using PLGA nanoparticles co-modified with cell-penetrating peptides and Engrailed secretion peptide (Sec), *Drug Deliv.* (2015) 1–12.
- [105] E. Dupont, A. Prochiantz, A. Joliot, Identification of a signal peptide for unconventional secretion, *J. Biol. Chem.* 282 (2007) 8994–9000.
- [106] J.E. Preston, N.J. Abbott, D.J. Begley, Chapter five—transcytosis of macromolecules at the blood–brain barrier, *Adv. Pharmacol.* 71 (2014) 147–163.
- [107] G.J. Doherty, H.T. McMahon, Mechanisms of endocytosis, *Annu. Rev. Biochem.* 78 (2009) 857–902.
- [108] J.T. Sockolovsky, M.R. Tiffany, F.C. Szoka, Engineering neonatal Fc receptor-mediated recycling and transcytosis in recombinant proteins by short terminal peptide extensions, *Proc. Natl. Acad. Sci.* 109 (2012) 16095–16100.
- [109] E.M. Pridgen, F. Alexis, T.T. Kuo, E. Levy-Nissenbaum, R. Karnik, R.S. Blumberg, R. Langer, O.C. Farokhzad, Transcellular transport of Fc-targeted nanoparticles by the neonatal fc receptor for oral delivery, *Sci. Transl. Med.* 5 (2013) 213ra167.
- [110] R. Rojas, G. Apodaca, Immunoglobulin transport across polarized epithelial cells, *Nat. Rev. Mol. Cell Biol.* 3 (2002) 944–956.
- [111] J.S. Wolf, G. Li, A. Varadhachary, K. Petrak, M. Schneyer, D. Li, J. Ongkasuwan, X. Zhang, R.J. Taylor, S.E. Strome, Oral lactoferrin results in T cell-dependent tumor inhibition of head and neck squamous cell carcinoma in vivo, *Clin. Cancer Res.* 13 (2007) 1601–1610.
- [112] G. Mahidhara, R.K. Kanwar, K. Roy, J.R. Kanwar, Oral administration of iron-saturated bovine lactoferrin-loaded ceramic nanocapsules for breast cancer therapy and influence on iron and calcium metabolism, *Int. J. Nanomedicine* 10 (2015) 4081.
- [113] C.H. Fazen, D. Valentin, T.J. Fairchild, R.P. Doyle, Oral delivery of the appetite suppressing peptide hPYY (3–36) through the vitamin B12 uptake pathway, *J. Med. Chem.* 54 (2011) 8707–8711.
- [114] D.H. Alpers, G. Russell-Jones, Gastric intrinsic factor: the gastric and small intestinal stages of cobalamin absorption. A personal journey, *Biochimie* 95 (2013) 989–994.
- [115] A. Verma, S. Sharma, P.K. Gupta, A. Singh, B.V. Teja, P. Dwivedi, G.K. Gupta, R. Trivedi, P.R. Mishra, Vitamin B12 functionalized layer by layer calcium phosphate nanoparticles: a mucoadhesive and pH responsive carrier for improved oral delivery of insulin, *Acta Biomater.* (2015).
- [116] K.B. Chalasani, G.J. Russell-Jones, A.K. Jain, P.V. Diwan, S.K. Jain, Effective oral delivery of insulin in animal models using vitamin B12-coated dextran nanoparticles, *J. Control. Release* 122 (2007) 141–150.
- [117] M. Cohen, D. Kitsberg, S. Tsytkin, M. Shulman, B. Aroeti, Y. Nahmias, Live imaging of GLUT2 glucose-dependent trafficking and its inhibition in polarized epithelial cells, *Open Biol.* 4 (2014) 140091.
- [118] C. Guan, J. Ji, C. Jin, G. Wang, X. Li, W. Guan, Expression of cholera toxin B subunit – lumbrokinase in edible sunflower seeds – the use of transmucosal carrier to enhance its fusion protein's effect on protection of rats and mice against thrombosis, *Biotechnol. Prog.* 30 (2014) 1029–1039.
- [119] N. Kohli, D.R. Westerveld, A.C. Ayache, A. Verma, P. Shil, T. Prasad, P. Zhu, S.L. Chan, Q. Li, H. Daniell, Oral delivery of bioencapsulated proteins across blood–brain and blood–retinal barriers, *Mol. Ther.* 22 (2014) 535–546.
- [120] K.L. Noakes, H.T. Teisserenc, J.M. Lord, P. Dunbar, V. Cerundolo, L.M. Roberts, Exploiting retrograde transport of Shiga-like toxin 1 for the delivery of exogenous antigens into the MHC class I presentation pathway, *FEBS Lett.* 453 (1999) 95–99.
- [121] J.-H. Ryou, Y.-K. Sohn, D.-E. Hwang, H.-S. Kim, Shiga-like toxin-based high-efficiency and receptor-specific intracellular delivery system for a protein, *Biochem. Biophys. Res. Commun.* 464 (2015) 1282–1289.
- [122] B.P. Hurley, M. Jacewicz, C. Thorpe, L.L. Lincicome, A.J. King, G.T. Keusch, D.W. Acheson, Shiga toxins 1 and 2 translocate differently across polarized intestinal epithelial cells, *Infect. Immun.* 67 (1999) 6670–6677.
- [123] Y.M. te Welscher, D.J.-F. Chinnapan, L. Kaoutzani, R.J. Mersny, W.I. Lencer, Unsaturated glyceramides as molecular carriers for mucosal drug delivery of GLP-1, *J. Control. Release* 175 (2014) 72–78.
- [124] G. Battogtokh, Y.T. Ko, Self-assembled chitosan-ceramide nanoparticle for enhanced oral delivery of paclitaxel, *Pharm. Res.* 31 (2014) 3019–3030.
- [125] N. Wang, T. Wang, M. Zhang, R. Chen, R. Niu, Y. Deng, Mannose derivative and lipid A dually decorated cationic liposomes as an effective cold chain free oral mucosal vaccine adjuvant-delivery system, *Eur. J. Pharm. Biopharm.* 88 (2014) 194–206.
- [126] W. Situ, X. Li, J. Liu, L. Chen, Preparation and characterization of glycoprotein-resistant starch complex as a coating material for oral bioadhesive microparticles for colon-targeted polypeptide delivery, *J. Agric. Food Chem.* 63 (2015) 4138–4147.
- [127] S. Mayor, R.G. Parton, J.G. Donaldson, Clathrin-independent pathways of endocytosis, *Cold Spring Harb. Perspect. Biol.* 6 (2014) a016758.
- [128] B. Ritter, S. Murphy, H. Dokainish, M. Girard, M.V. Gudheti, G. Kozlov, M. Halin, J. Philie, E.M. Jorgensen, K. Gehring, NECAP 1 regulates AP-2 interactions to control vesicle size, number, and cargo during clathrin-mediated endocytosis, *PLoS Biol.* 11 (2013), e1001670.
- [129] S.E. Miller, S. Mathiasen, N.A. Bright, F. Pierre, B.T. Kelly, N. Kladt, A. Schauss, C.J. Merrifield, D. Stamou, S. Höning, CALM regulates clathrin-coated vesicle size and maturation by directly sensing and driving membrane curvature, *Dev. Cell* 33 (2015) 163–175.
- [130] L. Pelkmans, A. Helenius, Endocytosis via caveolae, *Traffic* 3 (2002) 311–320.
- [131] S. Falcone, E. Cocucci, P. Podini, T. Kirchhausen, E. Clementi, J. Meldolesi, Macropinocytosis: regulated coordination of endocytic and exocytic membrane traffic events, *J. Cell Sci.* 119 (2006) 4758–4769.
- [132] D. Corda, C.H. Carcedo, M. Bonazzi, A. Luini, S. Spano, Molecular aspects of membrane fission in the secretory pathway, *Cell. Mol. Life Sci.* 59 (2002) 1819–1832.
- [133] G.J. Praefcke, H.T. McMahon, The dynamin superfamily: universal membrane tubulation and fission molecules? *Nat. Rev. Mol. Cell Biol.* 5 (2004) 133–147.
- [134] A. des Rieux, V. Pourcelle, P.D. Cani, J. Marchand-Brynaert, V. Prêt, Targeted nanoparticles with novel non-peptidic ligands for oral delivery, *Adv. Drug Deliv. Rev.* 65 (2013) 833–844.
- [135] X. Zhang, W. Wu, Ligand-mediated active targeting for enhanced oral absorption, *Drug Discov. Today* 19 (2014) 898–904.
- [136] R. Fowler, D. Vilasaliu, F.F. Trillo, M. Garnett, C. Alexander, H. Horsley, B. Smith, I. Whitcombe, M. Eaton, S. Stolnik, Nanoparticle transport in epithelial cells: pathway switching through bioconjugation, *Small* 9 (2013) 3282–3294.
- [137] A. des Rieux, E.G. Ragnarsson, E. Gullberg, V. Prêt, Y.-J. Schneider, P. Artursson, Transport of nanoparticles across an in vitro model of the human intestinal follicle associated epithelium, *Eur. J. Pharm. Sci.* 25 (2005) 455–465.
- [138] E. Gullberg, M. Leonard, J. Karlsson, A.M. Hopkins, D. Brayden, A.W. Baird, P. Artursson, Expression of specific markers and particle transport in a new human intestinal M-cell model, *Biochem. Biophys. Res. Commun.* 279 (2000) 808–813.
- [139] T. Kirchhausen, E. Macia, H.E. Pelish, Use of dynasore, the small molecule inhibitor of dynamin, in the regulation of endocytosis, *Methods Enzymol.* 438 (2008) 77–93.
- [140] E. Macia, M. Ehrlich, R. Massol, E. Boucrot, C. Brunner, T. Kirchhausen, Dynasore, a cell-permeable inhibitor of dynamin, *Dev. Cell* 10 (2006) 839–850.
- [141] A.I. Ivanov, Pharmacological inhibition of endocytic pathways: is it specific enough to be useful? *Exocytosis Endocytosis* (2008) 15–33.
- [142] D. Vercauteren, R.E. Vandembroucke, A.T. Jones, J. Rejman, J. Demeester, S.C. De Smedt, N.N. Sanders, K. Braeckmans, The use of inhibitors to study endocytic pathways of gene carriers: optimization and pitfalls, *Mol. Ther.* 18 (2010) 561–569.
- [143] H. Stenmark, Rab GTPases as coordinators of vesicle traffic, *Nat. Rev. Mol. Cell Biol.* 10 (2009) 513–525.
- [144] A.H. Hutagalung, P.J. Novick, Role of Rab GTPases in membrane traffic and cell physiology, *Physiol. Rev.* 91 (2011) 119–149.
- [145] E. Rodriguez-Boulan, I.G. Macara, Organization and execution of the epithelial polarity programme, *Nat. Rev. Mol. Cell Biol.* 15 (2014) 225–242.
- [146] W. Hunziker, P.J. Peters, Rab17 localizes to recycling endosomes and regulates receptor-mediated transcytosis in epithelial cells, *J. Biol. Chem.* 273 (1998) 15734–15741.
- [147] J.E. Casanova, X. Wang, R. Kumar, S.G. Bhartur, J. Navarre, J.E. Woodrum, Y. Altschuler, G.S. Ray, J.R. Goldenring, Association of Rab25 and Rab11a with the apical recycling system of polarized Madin-Darby canine kidney cells, *Mol. Biol. Cell* 10 (1999) 47–61.
- [148] D.R. Sheff, E.A. Daro, M. Hull, I. Mellman, The receptor recycling pathway contains two distinct populations of early endosomes with different sorting functions, *J. Cell Biol.* 145 (1999) 123–139.
- [149] P. Sandin, L.W. Fitzpatrick, J.C. Simpson, K.A. Dawson, High-speed imaging of Rab family small GTPases reveals rare events in nanoparticle trafficking in living cells, *ACS Nano* 6 (2012) 1513–1521.
- [150] O. Ullrich, S. Reinsch, S. Urbé, M. Zerial, R.G. Parton, Rab11 regulates recycling through the pericentriolar recycling endosome, *J. Cell Biol.* 135 (1996) 913–924.
- [151] M.E. Taub, W.-C. Shen, Polarity in the transcytotic processing of apical and basal membrane-bound peroxidase-polylysine conjugates in MDCK cells, *J. Cell. Physiol.* 150 (1992) 283–290.
- [152] W. He, M.S. Ladinsky, K.E. Huey-Tubman, G.J. Jensen, J.R. McIntosh, P.J. Björkman, FcRn-mediated antibody transport across epithelial cells revealed by electron tomography, *Nature* 455 (2008) 542–546.
- [153] M.E. Taub, W.-C. Shen, Regulation of pathways within cultured epithelial cells for the transcytosis of a basal membrane-bound peroxidase-polylysine conjugate, *J. Cell Sci.* 106 (1993) 1313–1322.
- [154] K. Prydz, S.H. Hansen, K. Sandvig, B. van Deurs, Effects of brefeldin A on endocytosis, transcytosis and transport to the Golgi complex in polarized MDCK cells, *J. Cell Biol.* 119 (1992) 259–272.
- [155] P.G. Cammisotto, M. Bendayan, A. Sané, M. Dominguez, C. Garofalo, E. Levy, Receptor-mediated transcytosis of leptin through human intestinal cells in vitro, *Int. J. Cell Biol.* 2010 (2010).
- [156] S.J., S.J. Mansour, X.H. Zhao, J. Giordano, S.W. Scherer, P. Melançon, p200 ARF-GEP1: a Golgi-localized guanine nucleotide exchange protein whose Sec7 domain is targeted by the drug brefeldin A, *Proc. Natl. Acad. Sci. U. S. A.* 96 (1999) 7968–7973.
- [157] J.G. Donaldson, D. Finazzi, R.D. Klausner, Brefeldin A Inhibits Golgi Membrane-catalysed Exchange of Guanine Nucleotide onto ARF Protein, 1992.

- [158] E.B. Cluett, S.A. Wood, M. Banta, W.J. Brown, Tubulation of Golgi membranes *in vivo* and *in vitro* in the absence of brefeldin A, *J. Cell Biol.* 120 (1993) 15–24.
- [159] K. Takashima, A. Saitoh, S. Hirose, W. Nakai, Y. Kondo, Y. Takasu, H. Kakeya, H.-W. Shin, K. Nakayama, GBF1-Arf-COPI-ArfGAP-mediated Golgi-to-ER transport involved in regulation of lipid homeostasis, *Cell Struct. Funct.* 36 (2011) 223–235.
- [160] G. Strous, P. Van Kerkhof, G. Van Meer, S. Rijnboudt, W. Stoorvogel, Differential effects of brefeldin A on transport of secretory and lysosomal proteins, *J. Biol. Chem.* 268 (1993) 2341–2347.
- [161] J. Scheel, R. Pepperkok, M. Lowe, G. Griffiths, T.E. Kreis, Dissociation of coatamer from membranes is required for brefeldin A-induced transfer of Golgi enzymes to the endoplasmic reticulum, *J. Cell Biol.* 137 (1997) 319–333.
- [162] R. Guidi, L. Levi, S.F. Rouf, S. Puia, M. Rhen, T. Frisan, Salmonella enterica delivers its genotoxin through outer membrane vesicles secreted from infected cells, *Cell. Microbiol.* 15 (2013) 2034–2050.
- [163] A. El Baya, R. Linnemann, L. von Olleschik-Elbheim, H. Robenek, M. Schmidt, Endocytosis and retrograde transport of pertussis toxin to the Golgi complex as a prerequisite for cellular intoxication, *Eur. J. Cell Biol.* 73 (1997) 40–48.
- [164] D.J.-F. Chinnapen, H. Chinnapen, D. Saslowky, W.I. Lencer, Rafting with cholera toxin: endocytosis and trafficking from plasma membrane to ER, *FEMS Microbiol. Lett.* 266 (2007) 129–137.
- [165] W. Nakai, Y. Kondo, A. Saitoh, T. Naito, K. Nakayama, H.-W. Shin, ARF1 and ARF4 regulate recycling endosomal morphology and retrograde transport from endosomes to the Golgi apparatus, *Mol. Biol. Cell* 24 (2013) 2570–2581.
- [166] T. Su, D.M. Bryant, F. Luton, M. Vergés, S.M. Ulrich, K.C. Hansen, A. Datta, D.J. Eastburn, A.L. Burlingame, K.M. Shokat, A kinase cascade leading to Rab11-FIP5 controls transcytosis of the polymeric immunoglobulin receptor, *Nat. Cell Biol.* 12 (2010) 1143–1153.
- [167] K. Baker, S.-W. Qiao, T. Kuo, K. Kobayashi, M. Yoshida, W.I. Lencer, R.S. Blumberg, Immune and non-immune functions of the (not so) neonatal Fc receptor, *FcRn*, *Semin. Immunopathol.* 31 (2009) 223–236.
- [168] S. Tzaban, R.H. Massol, E. Yen, W. Hamman, S.R. Frank, L.A. Lapiere, S.H. Hansen, J.R. Goldenring, R.S. Blumberg, W.I. Lencer, The recycling and transcytotic pathways for IgG transport by FcRn are distinct and display an inherent polarity, *J. Cell Biol.* 185 (2009) 673–684.
- [169] B.L. Dickinson, K. Badizadegan, Z. Wu, J.C. Ahouse, X. Zhu, N.E. Simister, R.S. Blumberg, W.I. Lencer, Bidirectional FcRn-dependent IgG transport in a polarized human intestinal epithelial cell line, *J. Clin. Invest.* 104 (1999) 903–911.
- [170] Z. Wu, N.E. Simister, Tryptophan- and dileucine-based endocytosis signals in the neonatal Fc receptor, *J. Biol. Chem.* 276 (2001) 5240–5247.
- [171] O. Pabst, A. Mowat, Oral tolerance to food protein, *Mucosal Immunol.* 5 (2012) 232–239.
- [172] T. Rath, T.T. Kuo, K. Baker, S.-W. Qiao, K. Kobayashi, M. Yoshida, D. Roopenian, E. Fiebiger, W.I. Lencer, R.S. Blumberg, The immunologic functions of the neonatal Fc receptor for IgG, *J. Clin. Immunol.* 33 (2013) 9–17.
- [173] A.J. Bitonti, J.A. Dumont, Pulmonary administration of therapeutic proteins using an immunoglobulin transport pathway, *Adv. Drug Deliv. Rev.* 58 (2006) 1106–1118.
- [174] J.A. Dumont, A.J. Bitonti, D. Clark, S. Evans, M. Pickford, S.P. Newman, Delivery of an erythropoietin-Fc fusion protein by inhalation in humans through an immunoglobulin transport pathway, *J. Aerosol Med.* 18 (2005) 294–303.
- [175] D. Vilasaliu, C. Alexander, M. Garnett, M. Eaton, S. Stolnik, Fc-mediated transport of nanoparticles across airway epithelial cell layers, *J. Control. Release* 158 (2012) 479–486.
- [176] J.C. Fyfe, M. Madsen, P. Højrup, E.I. Christensen, S.M. Tanner, A. de la Chapelle, Q. He, S.K. Moestrup, The functional cobalamin (vitamin B12)-intrinsic factor receptor is a novel complex of cubilin and amnionless, *Blood* 103 (2004) 1573–1579.
- [177] G.A. Pedersen, S. Chakraborty, A.L. Steinhilber, L.M. Traub, M. Madsen, AMN directs endocytosis of the intrinsic factor-vitamin B12 receptor cubam by engaging ARH or Dab2, *Traffic* 11 (2010) 706–720.
- [178] D.H. Alpers, Absorption and blood/cellular transport of folate and cobalamin: pharmacokinetic and physiological considerations, *Biochimie* (2015).
- [179] L.H. Allen, How common is vitamin B-12 deficiency? *Am. J. Clin. Nutr.* 89 (2009) 693S–696S.
- [180] H. He, P. Wang, C. Cai, R. Yang, X. Tang, VB 12-coated Gel-Core-SLN containing insulin: another way to improve oral absorption, *Int. J. Pharm.* 493 (2015) 451–459.
- [181] U. Wrackmeyer, G.H. Hansen, T. Seya, E.M. Danielsen, Intelectin: a novel lipid raft-associated protein in the enterocyte brush border, *Biochemistry* 45 (2006) 9188–9197.
- [182] B. Lönnerdal, R. Jiang, X. Du, Bovine lactoferrin can be taken up by the human intestinal lactoferrin receptor and exert bioactivities, *J. Pediatr. Gastroenterol. Nutr.* 53 (2011) 606–614.
- [183] R. Jiang, V. Lopez, S.L. Kelleher, B. Lönnerdal, Apo- and holo-lactoferrin are both internalized by lactoferrin receptor via clathrin-mediated endocytosis but differentially affect ERK-signaling and cell proliferation in caco-2 cells, *J. Cell. Physiol.* 226 (2011) 3022–3031.
- [184] R. Prades, B. Oller-Salvia, S.M. Schwarzmaier, J. Selva, M. Moros, M. Balbi, V. Grazú, J.M. de La Fuente, G. Egea, N. Plesnila, Applying the retro-enantio approach to obtain a peptide capable of overcoming the blood-brain barrier, *Angew. Chem. Int. Ed.* 54 (2015) 3967–3972.
- [185] S. Gulec, G.J. Anderson, J.F. Collins, Mechanistic and regulatory aspects of intestinal iron absorption, *Am. J. Physiol. Gastrointest. Liver Physiol.* 307 (2014) G397–G409.
- [186] W.J. Griffiths, T.M. Cox, Co-localization of the mammalian hemochromatosis gene product (HFE) and a newly identified transferrin receptor (TfR2) in intestinal tissue and cells, *J. Histochem. Cytochem.* 51 (2003) 613–623.
- [187] W. Zheng, J. Kollmeyer, H. Symolon, A. Momin, E. Munter, E. Wang, S. Kelly, J.C. Allegood, Y. Liu, Q. Peng, Ceramides and other bioactive sphingolipid backbones in health and disease: lipidomic analysis, metabolism and roles in membrane structure, dynamics, signaling and autophagy, *Biochim. Biophys. Acta Biomembr.* 1758 (2006) 1864–1884.
- [188] Y.H. Zeidan, Y.A. Hannun, Translational aspects of sphingolipid metabolism, *Trends Mol. Med.* 13 (2007) 327–336.
- [189] K. Hanada, K. Kumagai, S. Yasuda, Y. Miura, M. Kawano, M. Fukasawa, M. Nishijima, Molecular machinery for non-vesicular trafficking of ceramide, *Nature* 426 (2003) 803–809.
- [190] D.J.-F. Chinnapen, W.-T. Hsieh, Y.M. te Welscher, D.E. Saslowky, L. Kaoutzani, E. Brandsma, L. D'Auria, H. Park, J.S. Wagner, K.R. Drake, Lipid sorting by ceramide structure from plasma membrane to ER for the cholera toxin receptor ganglioside GM1, *Dev. Cell* 23 (2012) 573–586.
- [191] D.E. Saslowky, Y.M. te Welscher, D.J.-F. Chinnapen, J.S. Wagner, J. Wan, E. Kern, W.I. Lencer, Ganglioside GM1-mediated transcytosis of cholera toxin bypasses the retrograde pathway and depends on the structure of the ceramide domain, *J. Biol. Chem.* 288 (2013) 25804–25809.
- [192] J.J. Powell, E. Thomas-McKay, V. Thoree, J. Robertson, R.E. Hewitt, J.N. Skepper, A. Brown, J.C. Hernandez-Garrido, P.A. Midgley, I. Gomez-Morilla, An endogenous nanomineral chaperones luminal antigen and peptidoglycan to intestinal immune cells, *Nat. Nanotechnol.* (2015).
- [193] J.D. Söderholm, Gut immunology: nanoparticles ferry gut antigens, *Nat. Nanotechnol.* 10 (2015) 298–299.
- [194] M.R. Neutra, N.J. Mantis, J.-P. Kraehenbuhl, Collaboration of epithelial cells with organized mucosal lymphoid tissues, *Nat. Immunol.* 2 (2001) 1004–1009.
- [195] C. Wallon, Y. Braaf, M. Wolving, G. Olaison, J.D. Söderholm, Endoscopic biopsies in Ussing chambers evaluated for studies of macromolecular permeability in the human colon, *Scand. J. Gastroenterol.* 40 (2005) 586–595.
- [196] M. Foltz, A. Cerstiaens, A. van Meensel, R. Mols, P.C. van der Pijl, G.S. Duchateau, P. Augustijns, The angiotensin converting enzyme inhibitory tripeptides Ile-Pro-Pro and Val-Pro-Pro show increasing permeabilities with increasing physiological relevance of absorption models, *Peptides* 29 (2008) 1312–1320.
- [197] H.J. Van Kruiningen, A.B. West, B.J. Freda, K.A. Holmes, Distribution of Peyer's patches in the distal ileum, *Inflamm. Bowel Dis.* 8 (2002) 180–185.
- [198] H. Van Kruiningen, L. Ganley, B. Freda, The role of Peyer's patches in the age-related incidence of Crohn's disease, *J. Clin. Gastroenterol.* 25 (1997) 470–475.
- [199] P.C. Tyrer, A.R. Foxwell, J.M. Kyd, D.C. Otczyk, A.W. Cripps, Receptor mediated targeting of M-cells, *Vaccine* 25 (2007) 3204–3209.
- [200] J.-P. Kraehenbuhl, M.R. Neutra, Epithelial M cells: differentiation and function, *Annu. Rev. Cell Dev. Biol.* 16 (2000) 301–332.
- [201] E. Gullberg, Å.V. Keita, Y.S. Sa'ad, M. Andersson, K.D. Caldwell, J.D. Söderholm, P. Artursson, Identification of cell adhesion molecules in the human follicle-associated epithelium that improve nanoparticle uptake into the Peyer's patches, *J. Pharmacol. Exp. Ther.* 319 (2006) 632–639.
- [202] J. Soni, A.W. Baird, L.M. O'Brien, M. McElroy, J.J. Callanan, H.F. Bassett, D. Campion, D.J. Brayden, Rat, ovine and bovine Peyer's patches mounted in horizontal diffusion chambers display sampling function, *J. Control. Release* 115 (2006) 68–77.
- [203] N.N. Parayath, H. Nehoff, P. Mueller, S. Taurin, K. Greish, Styrene maleic acid micelles as a nanocarrier system for oral anticancer drug delivery—dual uptake through enterocytes and M-cells, *Int. J. Nanomedicine* 10 (2015) 4653.
- [204] C. Damgå, M. Aprahamian, W. Humbert, M. Pinget, Ileal uptake of polyalkylcyanoacrylate nanocapsules in the rat, *J. Pharm. Pharmacol.* 52 (2000) 1049–1056.
- [205] J. Pappo, T. Ermak, Uptake and translocation of fluorescent latex particles by rabbit Peyer's patch follicle epithelium: a quantitative model for M cell uptake, *Clin. Exp. Immunol.* 76 (1989) 144.
- [206] M. De Jesus, G.R. Ostroff, S.M. Levitz, T.R. Bartling, N.J. Mantis, A population of Langerin-positive dendritic cells in murine Peyer's patches involved in sampling  $\beta$ -glucan microparticles, *PLoS One* 9 (2014), e91002.
- [207] V.K. Shreedhar, B.L. Kelsall, M.R. Neutra, Cholera toxin induces migration of dendritic cells from the subepithelial dome region to T- and B-cell areas of Peyer's patches, *Infect. Immun.* 71 (2003) 504–509.
- [208] E. Lawless, B.T. Griffin, A. O'Mahony, C.M. O'Driscoll, Exploring the impact of drug properties on the extent of intestinal lymphatic transport—in vitro and in vivo studies, *Pharm. Res.* 32 (2015) 1817–1829.
- [209] H. YOSHIKAWA, K. TAKADA, S. MURANISHI, Molecular weight dependence of permeability to rat small intestinal blood-lymph barrier for exogenous macromolecules absorbed from lumen, *J. Pharmacobiodyn.* 7 (1984) 1–6.
- [210] D.A. Rothenfluh, H. Bermudez, C.P. O'Neil, J.A. Hubbell, Biofunctional polymer nanoparticles for intra-articular targeting and retention in cartilage, *Nat. Mater.* 7 (2008) 248–254.
- [211] P.B. Patil, P.B. Chougule, V.K. Kumar, S. Almström, H. Bäckdahl, D. Banerjee, G. Herlenius, M. Olsson, S. Sumitran-Holgersson, Recellularization of acellular human small intestine using bone marrow stem cells, *Stem Cells Transl. Med.* (2013), <http://dx.doi.org/10.5966/sctm.2012-0108>.
- [212] C.A. Barnes, J. Brison, R. Michel, B.N. Brown, D.G. Castner, S.F. Badyal, B.D. Ratner, The surface molecular functionality of decellularized extracellular matrices, *Biomaterials* 32 (2011) 137–143.
- [213] P. Artursson, Epithelial transport of drugs in cell culture. I: a model for studying the passive diffusion of drugs over intestinal absorptive (Caco-2) cells, *J. Pharm. Sci.* 79 (1990) 476–482.
- [214] G. Wilson, I.F. Hassan, C.J. Dix, I. Williamson, R. Shah, M. Mackay, P. Artursson, Transport and permeability properties of human Caco-2 cells: an in vitro model of the intestinal epithelial cell barrier, *J. Control. Release* 11 (1990) 25–40.
- [215] A.R. Hilgers, R.A. Conradi, P.S. Burton, Caco-2 cell monolayers as a model for drug transport across the intestinal mucosa, *Pharm. Res.* 7 (1990) 902–910.

- [216] I.J. Hidalgo, T.J. Raub, R.T. Borchardt, Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability, *Gastroenterology* 96 (1989) 736–749.
- [217] I. Hubatsch, E.G. Ragnarsson, P. Artursson, Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers, *Nat. Protoc.* 2 (2007) 2111–2119.
- [218] S. Tavelin, J. Gråsjö, J. Taipalensuu, G. Ocklind, P. Artursson, Applications of epithelial cell culture in studies of drug transport, *Epithelial Cell Cult. Protoc.* (2002) 233–272.
- [219] A. Wikman, J. Karlsson, I. Carlstedt, P. Artursson, A drug absorption model based on the mucus layer producing human intestinal goblet cell line HT29-H, *Pharm. Res.* 10 (1993) 843–852.
- [220] J. Karlsson, A. Wikman, P. Artursson, The mucus layer as a barrier to drug absorption in monolayers of human intestinal epithelial HT29-H goblet cells, *Int. J. Pharm.* 99 (1993) 209–218.
- [221] C. Hilgendorf, H. Spahn-Langguth, C.G. Regårdh, E. Lipka, G.L. Amidon, P. Langguth, Caco-2 versus caco-2/HT29-MTX co-cultured cell lines: permeabilities via diffusion, inside-and outside-directed carrier-mediated transport, *J. Pharm. Sci.* 89 (2000) 63–75.
- [222] A. Wikman-Larhed, P. Artursson, Co-cultures of human intestinal goblet (HT29-H) and absorptive (Caco-2) cells for studies of drug and peptide absorption, *Eur. J. Pharm. Sci.* 3 (1995) 171–183.
- [223] C. Schimpel, B. Teubl, M. Absenger, C. Meindl, E. Fröhlich, G. Leitinger, A. Zimmer, E. Roblegg, Development of an advanced intestinal in vitro triple culture permeability model to study transport of nanoparticles, *Mol. Pharm.* 11 (2014) 808–818.
- [224] I. Behrens, P. Stenberg, P. Artursson, T. Kissel, Transport of lipophilic drug molecules in a new mucus-secreting cell culture model based on HT29-MTX cells, *Pharm. Res.* 18 (2001) 1138–1145.
- [225] S. Keely, A. Rullay, C. Wilson, A. Carmichael, S. Carrington, A. Corfield, D.M. Haddleton, D.J. Brayden, In vitro and ex vivo intestinal tissue models to measure mucoadhesion of poly (methacrylate) and N-trimethylated chitosan polymers, *Pharm. Res.* 22 (2005) 38–49.
- [226] J. Griebinger, S. Dünnhaupt, B. Cattoz, P. Griffiths, S. Oh, S.B.I. Gómez, M. Wilcox, J. Pearson, M. Gumbleton, M. Abdulkarim, Methods to determine the interactions of micro- and nanoparticles with mucus, *Eur. J. Pharm. Biopharm.* 96 (2015) 464–476.
- [227] A.F. Quest, J.L. Gutierrez-Pajares, V.A. Torres, Caveolin-1: an ambiguous partner in cell signalling and cancer, *J. Cell. Mol. Med.* 12 (2008) 1130–1150.
- [228] S. Hehlhans, N. Cordes, Caveolin-1: an essential modulator of cancer cell radio- and chemoresistance, *Am. J. Cancer Res.* 1 (2011) 521–530.
- [229] N. Dasgupta, B.K. Thakur, A. Ta, S. Das, Caveolin-1 is transcribed from a hypermethylated promoter to mediate colonocyte differentiation and apoptosis, *Exp. Cell Res.* 334 (2015) 323–336.
- [230] P. Ilina, S. Partti, J. Niklander, M. Ruponen, Y.-R. Lou, M. Yliperttula, Effect of differentiation on endocytic profiles of endothelial and epithelial cell culture models, *Exp. Cell Res.* 332 (2015) 89–101.
- [231] S. Kernéis, A. Bogdanova, J.-P. Kraehenbuhl, E. Pringault, Conversion by Peyer's patch lymphocytes of human enterocytes into M cells that transport bacteria, *Science* 277 (1997) 949–952.
- [232] M. Miyake, E. Ragnarsson, D. Nakai, P. Artursson, The pro-inflammatory cytokine interleukin-6 regulates nanoparticle transport across model follicle-associated epithelium cells, *J. Pharm. Sci.* (2016).
- [233] E.G. Ragnarsson, I. Schoultz, E. Gullberg, A.H. Carlsson, F. Tafazolli, M. Lerm, K.-E. Magnusson, J.D. Söderholm, P. Artursson, *Yersinia pseudotuberculosis* induces transcytosis of nanoparticles across human intestinal villus epithelium via invasion-dependent macropinosytosis, *Lab. Invest.* 88 (2008) 1215–1226.
- [234] K. Dharmasathaphorn, S. Carlson, Structural analysis of a human intestinal epithelial cell line, *Gastroenterology* 92 (1987) 1133–1145.
- [235] S. Tavelin, V. Milovic, G. Ocklind, S. Olsson, P. Artursson, A conditionally immortalized epithelial cell line for studies of intestinal drug transport, *J. Pharmacol. Exp. Ther.* 290 (1999) 1212–1221.
- [236] E. Paul, J. Hochman, A. Quaroni, Conditionally immortalized intestinal epithelial cells: novel approach for study of differentiated enterocytes, *Am. J. Phys. Cell Phys.* 265 (1993) C266–C278.
- [237] T. Takenaka, N. Harada, J. Kuze, M. Chiba, T. Iwao, T. Matsunaga, Human small intestinal epithelial cells differentiated from adult intestinal stem cells as a novel system for predicting oral drug absorption in humans, *Drug Metab. Dispos.* 42 (2014) 1947–1954.
- [238] T. Takenaka, N. Harada, J. Kuze, M. Chiba, T. Iwao, T. Matsunaga, Application of a human intestinal epithelial cell monolayer to the prediction of oral drug absorption in humans as a superior alternative to the caco-2 cell monolayer, *J. Pharm. Sci.* 105 (2016) 915–924.
- [239] N. Navabi, M.A. McGuckin, S.K. Lindén, Gastrointestinal cell lines form polarized epithelia with an adherent mucus layer when cultured in semi-wet interfaces with mechanical stimulation, *PLoS One* 8 (2013), e68761.
- [240] S.H. Kim, M. Chi, B. Yi, S.H. Kim, S. Oh, Y. Kim, S. Park, J.H. Sung, Three-dimensional intestinal villi epithelium enhances protection of human intestinal cells from bacterial infection by inducing mucin expression, *Integr. Biol.* 6 (2014) 1122–1131.
- [241] S.N. Bhatia, D.E. Ingber, Microfluidic organs-on-chips, *Nature* 201 (2014) 4.
- [242] H.J. Kim, D.E. Ingber, Gut-on-a-Chip microenvironment induces human intestinal cells to undergo villus differentiation, *Integr. Biol.* 5 (2013) 1130–1140.
- [243] H.J. Kim, D. Huh, G. Hamilton, D.E. Ingber, Human gut-on-a-chip inhabited by microbial flora that experiences intestinal peristalsis-like motions and flow, *Lab Chip* 12 (2012) 2165–2174.
- [244] P.B. Persson, Ussing's chamber origin, *Acta Physiol.* (2016).
- [245] S.M. Kalman, H.H. Ussing, Active sodium uptake by the toad and its response to the antidiuretic hormone, *J. Gen. Physiol.* 38 (1955) 361–370.
- [246] C. Schmidt, C. Lautenschlaeger, E.-M. Collnot, M. Schumann, C. Bojarski, J.-D. Schulzke, C.-M. Lehr, A. Stallmach, Nano- and microscaled particles for drug targeting to inflamed intestinal mucosa—a first in vivo study in human patients, *J. Control. Release* 165 (2013) 139–145.
- [247] C. Lautenschläger, C. Schmidt, C.-M. Lehr, D. Fischer, A. Stallmach, PEG-functionalized microparticles selectively target inflamed mucosa in inflammatory bowel disease, *Eur. J. Pharm. Biopharm.* 85 (2013) 578–586.
- [248] M. Ng, T. Fleming, M. Robinson, B. Thomson, N. Graetz, C. Margono, E.C. Mullany, S. Biryukov, C. Abbafati, S.F. Abera, Global, regional, and national prevalence of overweight and obesity in children and adults during 1980–2013: a systematic analysis for the Global Burden of Disease Study 2013, *Lancet* 384 (2014) 766–781.
- [249] T.F. Teixeira, M.C. Collado, C.L. Ferreira, J. Bressan, Mdo C. Peluzio, Potential mechanisms for the emerging link between obesity and increased intestinal permeability, *Nutr. Res.* 32 (2012) 637–647.
- [250] S. Ding, P.K. Lund, Role of intestinal inflammation as an early event in obesity and insulin resistance, *Curr. Opin. Nutr. Metab. Care* 14 (2011) 328.
- [251] H.E. Raybould, Gut microbiota, epithelial function and derangements in obesity, *J. Physiol.* 590 (2012) 441–446.
- [252] E. Lau, C. Marques, D. Pestana, M. Santoalha, D. Carvalho, P. Freitas, C. Calhau, The role of I-FABP as a biomarker of intestinal barrier dysfunction driven by gut microbiota changes in obesity, *Nutr. Metab.* 13 (2016) 1.
- [253] C. Kless, V.M. Müller, V.L. Schüppel, M. Lichtenegger, M. Rychlik, H. Daniel, M. Klingenspor, D. Haller, Diet-induced obesity causes metabolic impairment independent of alterations in gut barrier integrity, *Mol. Nutr. Food Res.* 59 (2015) 968–978.
- [254] A. Farhadi, S. Gundlapalli, M. Shaikh, C. Frantzides, L. Harrell, M.M. Kwasny, A. Keshavarzian, Susceptibility to gut leakiness: a possible mechanism for endotoxaemia in non-alcoholic steatohepatitis, *Liver Int.* 28 (2008) 1026–1033.
- [255] Å. Sjöberg, M. Lutz, C. Tannergren, C. Wingolf, A. Borde, A.-L. Ungell, Comprehensive study on regional human intestinal permeability and prediction of fraction absorbed of drugs using the Ussing chamber technique, *Eur. J. Pharm. Sci.* 48 (2013) 166–180.
- [256] A.H. Gitter, M. Fromm, J.-D. Schulzke, Impedance analysis for the determination of epithelial and subepithelial resistance in intestinal tissues, *J. Biochem. Biophys. Methods* 37 (1998) 35–46.
- [257] L.L. Clarke, A guide to Ussing chamber studies of mouse intestine, *Am. J. Physiol. Gastrointest. Liver Physiol.* 296 (2009) G1151–G1166.
- [258] J. Reis, A. DeZani, T. Pereira, A. Avdeef, C. Serra, Lamivudine permeability study: a comparison between PAMPA, ex vivo and in situ Single-Pass Intestinal Perfusion (SPIP) in rat jejunum, *Eur. J. Pharm. Sci.* 48 (2013) 781–789.
- [259] B. McNamara, D.C. Winter, J.E. Cuffe, G.C. O'Sullivan, B.J. Harvey, Basolateral K<sup>+</sup> channel involvement in forskolin-activated chloride secretion in human colon, *J. Physiol.* 519 (1999) 251–260.
- [260] N.G. Schipper, K.M. Vårum, P. Stenberg, G. Ocklind, H. Lennernäs, P. Artursson, Chitosans as absorption enhancers of poorly absorbable drugs: 3: influence of mucus on absorption enhancement, *Eur. J. Pharm. Sci.* 8 (1999) 335–343.
- [261] L.-A.B. Rawlinson, P.J. O'Brien, D.J. Brayden, High content analysis of cytotoxic effects of pDMAEMA on human intestinal epithelial and monocyte cultures, *J. Control. Release* 146 (2010) 84–92.
- [262] D.J. Brayden, S.-A. Cryan, K.A. Dawson, P.J. O'Brien, J.C. Simpson, High-content analysis for drug delivery and nanoparticle applications, *Drug Discov. Today* 20 (2015) 942–957.
- [263] E.G. van de Kerkhof, A.-L.B. Ungell, Å.K. Sjöberg, M.H. de Jager, C. Hilgendorf, I.A. de Graaf, G.M. Groothuis, Innovative methods to study human intestinal drug metabolism in vitro: precision-cut slices compared with ussing chamber preparations, *Drug Metab. Dispos.* 34 (2006) 1893–1902.
- [264] V. Rozehnal, D. Nakai, U. Hoepner, T. Fischer, E. Kamiyama, M. Takahashi, S. Yasuda, J. Mueller, Human small intestinal and colonic tissue mounted in the Ussing chamber as a tool for characterizing the intestinal absorption of drugs, *Eur. J. Pharm. Sci.* 46 (2012) 367–373.
- [265] P.J. Hornby, P.R. Cooper, C. Kliwinski, E. Ragwan, J.R. Mabus, B. Harman, S. Thompson, A.L. Kauffman, Z. Yan, S.H. Tam, Human and non-human primate intestinal FcRn expression and immunoglobulin G transcytosis, *Pharm. Res.* 31 (2014) 908–922.
- [266] P. Calleja, S. Espuelas, C. Vauthier, G. Ponchel, J.M. Irache, Controlled release, intestinal transport, and oral bioavailability of paclitaxel can be considerably increased using suitably tailored pegylated poly (anhydride) nanoparticles, *J. Pharm. Sci.* 104 (2015) 2877–2886.
- [267] J. Chen, C. Liu, W. Shan, Z. Xiao, H. Guo, Y. Huang, Enhanced stability of oral insulin in targeted peptide ligand trimethyl chitosan nanoparticles against trypsin, *J. Microencapsul.* 32 (2015) 632–641.
- [268] T. Fan, C. Chen, H. Guo, J. Xu, J. Zhang, X. Zhu, Y. Yang, Z. Zhou, L. Li, Y. Huang, Design and evaluation of solid lipid nanoparticles modified with peptide ligand for oral delivery of protein drugs, *Eur. J. Pharm. Biopharm.* 88 (2014) 518–528.
- [269] H. Lennernäs, Ö. Ahrenstedt, R. Hällgren, L. Knutson, M. Ryde, L.K. Paalzow, Regional jejunal perfusion, a new in vivo approach to study oral drug absorption in man, *Pharm. Res.* 9 (1992) 1243–1251.