Effects of lifestyle and environmental factors on intestinal epithelial cell morphogenesis
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Chapter 1

Introduction & scope of the thesis

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The intestinal epithelium is a polarized cell monolayer which is crucial for its function

The intestinal epithelium is a 1-cell-thick layer (a monolayer) separating the lumen of the gut from the rest of the body. The intestinal epithelium functions as the gateway between these compartments; taking up fluids and nutrients from the gut, and preventing these to leak into the lumen, thus performing the main task of the gut.

In order to form a functional tissue, individual cells must organize themselves in context of each other and their surroundings, in a process termed ‘morphogenesis’; from the Greek ‘beginning of the shape’. To be able to organize as a tissue, (intestinal) epithelial cells adhere to their neighbor cells, utilizing transmembrane proteins. The mature intestinal epithelial cells are, like all epithelial cells, polarized, i.e. display an asymmetric distribution of cell components allowing for different functions on different locations in the cells. IECs have distinct membrane domains, an ‘apical’ domain facing the intestinal lumen, and a ‘basolateral’ domain facing away from the lumen, with the lateral part facing and contacting the neighboring IECs, and the basal part facing and contacting the underlying tissue. The lateral surface houses intercellular adhesions, including E-cadherin-based adherens junctions and Claudin-based tight junctions, which ensure monolayer strength and impermeability, respectively. The basal part harbors transmembrane proteins attaching the IEC to the basement membrane, produced by the IECs themselves and local fibroblasts. The apical and basolateral domains are separated by tight junctions (TJs) sealing the intercellular space. TJ localization is part of apical-basal polarity; and prevents diffusion of membrane proteins between the apical and basolateral plasma membrane domains it separates. The TJs, by virtue of the pore-forming Claudins, are rate limiting for...
epithelial permeability of electrolytes and water\textsuperscript{6-8} and restrict the localization of secreted molecules in either gut lumen or lamina propria\textsuperscript{9,10}. Tight junctions also recruit transcription factors and prevent their translocation to the nucleus, and in this way contribute to the regulation of cell proliferation\textsuperscript{11}. The apical membrane of intestinal epithelial cells harbors numerous densely and uniformly packed finger-like projections filled with actin filament bundles tethered to the subapical actin web, called microvilli. Microvilli are essential for enterocyte function; enlarging the apical absorptive area 6- to 15- fold while minimally influencing cellular volume\textsuperscript{12,13}.

Polarity of IECs is initiated and maintained by the intracellular trafficking machinery\textsuperscript{14}. This epithelial polarity program (further detailed in the next chapter) consists of three evolutionary conserved polarity complexes; one consisting of Crb3a, Pals1 and PATJ, one consisting of Par3, Par6, atypical (a)PKC and the small GTPase Cdc42, and one consisting of Lgl, Dlg and Scribble\textsuperscript{14}. Dysregulation of these polarity complexes has been correlated with impaired intestinal epithelial morphogenesis and homeostasis\textsuperscript{15-18}.

IECs originate from stem cells that reside in crypts, which are invaginations in the intestinal wall. These stem cells give rise to highly proliferative IECs that differentiate and polarize as they migrate from the crypts to the surface epithelium (in the colon) or to the villi (in the small intestine), were they are eventually shed into the lumen. This morphogenic process is tightly controlled in time (3-4 days) and space to ensure maintenance of monolayer organization and, consequently, an adequate barrier function. In order for the monolayer to remain intact and in contact with the basement membrane, every cell that divides must maintain its polarized configuration and divide in a planar orientation, i.e. perpendicular to the apical-basal axis\textsuperscript{19-21}.
Concluding, specific cellular characteristics are crucial for epithelial morphogenesis and therefore function. These characteristics are the capacity to (1) engage in cohesive interactions with neighbor cells, (2) create distinct apical and basolateral membrane domains separated by tight junctions, (3) establish a polarized distribution of cellular components, and (4) to maintain their spatial relationship to the environment, i.e., remain in a monolayer (planar orientation) separating lumen from the underlying tissue1 Fig 1.

**External factors disrupt epithelial barrier function**

Inflammatory bowel diseases (IBD), encompassing Crohn’s disease (CD) and ulcerative colitis (UC), are characterized by a chronic inflammation of the intestine, relapsing diarrhoea, and additional symptoms such as vomiting, abdominal pain, and weight loss. UC is limited to the colon, were it affects the superficial mucosal layer22. CD is not limited to any part of the gastro-intestinal tract, but is predominant in the ileocecal region, and is characterized by transmural ulcers23. The aetiology of IBD is not fully elucidated. The concordance between monozygotic twins is 20-50% for CD and 16% for UC, implying a significant role for environmental (extrinsic) factors. Extrinsic, i.e., non-epithelium derived factors include smoking tobacco, appendectomy, hygiene as per the ‘hygiene hypothesis24, infectious pathogens and antibiotics, other medications such as aspirins or NSAIDs, a lack of fibers in diet, major life stressors, anxiety and depression25. These factors can be classified as ‘western lifestyle’. Indeed, the incidence of IBD has increased in the last decades in the western world, as well as in countries adapting to western lifestyle26,27. Amongst life style factors especially smoking
Figure 1 Schematic overview of typical polarized intestinal epithelium showing different membrane domains separated by tight junctions (TJ), the apical domain harboring microvilli and facing the intestinal lumen, and the basolateral membrane facing extracellular matrix and basolateral membranes of neighbor cell, to which it is anchored with adherens junctions (AJ). Also depicted is the theoretic apicobasal axis, perpendicular to which cell division takes place in order to maintain the monolayer.
stands out; with smokers having a higher change of developing CD and a lower change of developing UC relative to non-smokers\textsuperscript{28,29}. While IBD was emerging it seemed a disease disproportionally afflicting people with higher socio-economic status, which is seemingly at odds with their generally lesser exposition to above stated extrinsic factors; however, socio-economic status as an independent predictor has not been uniformly found in recent research\textsuperscript{30}.

On tissue and cellular level IBD is characterized by mucosal and epithelial injury and increased permeability\textsuperscript{31-33}, including changes in epithelial tight junctions, mucosal lesions, epithelial restoration failure, and changed functionality of the epithelial cells, which are correlated with immune deregulation\textsuperscript{34}. Local inflammatory mediators and lifestyle factors play an important role in this perturbation of epithelial function. Increased expression of pro-inflammatory cytokines (i.e. such as interferon gamma (IFN\gamma) and Tumor Necrosis Factor (TNF), IL-1\beta, IL-6, IL-17, IL-22, IL-23) plays a crucial role in the perturbation of epithelial function in IBD\textsuperscript{33,35-37}. IFN\gamma and TNF directly target and negatively affect intestinal epithelial cells. For instance, IFN\gamma and TNF disrupt a previously intact intestinal epithelial barrier in a synergistic manner by eliciting intracellular signalling pathways that negatively affect tight junctions that control paracellular transport\textsuperscript{38-40} and control the release of transcription factors\textsuperscript{41,42}. The importance of cytokines in the pathogenesis of IBD is further underscored by the effectiveness of anti-TNF monoclonal antibodies in the treatment of IBD in the clinic\textsuperscript{37,43}. The effectiveness of anti-TNF monoclonal antibodies could not be found with recombinant TNF-receptors, possibly because of a less stable bond, or because of a not fully elucidated enhanced pro-inflammatory cytokine production\textsuperscript{44-48}. Cigarette smoking as a Life style factor has been
found to negatively affect intestinal epithelial function in animal models. These studies reveal a causal relationship between the breathing in of cigarette smoke and therefore basal exposition to IECs (via blood vessels), and the effects on morphogenesis. In this way, cigarette smoke exposure impaired intestinal barrier function\textsuperscript{49,50}, increased apoptosis and autophagy in the ileum\textsuperscript{51,52}, decreased cystic fibrosis transmembrane conductance regulator (CFTR) activity in the intestinal epithelium\textsuperscript{53}, and increased bacterial translocation, intestinal villi atrophy\textsuperscript{49}, and altered tight junction protein expression in a small/large intestine-specific fashion\textsuperscript{49,54}. In cell models, exposure to cigarette smoke extract has been demonstrated to perturb epithelial barrier function and TJs in pulmonic epithelia cells\textsuperscript{55,56} and, likewise, impaired intestinal barrier function has been associated with the smoking-related lung disease chronic obstructive pulmonary disease (COPD)\textsuperscript{50,57}

As the intestinal epithelium is the first defense layer against whatever is in the gut, it is prone to damage by mechanical or immune-mediated stressors. If a patch of epithelium is damaged; functionality is lost. Epithelial wound healing consists of three different processes; migration of dedifferentiated epithelium, ‘restitution’, subsequent proliferation, and the final morphogenetic step of differentiation and thereby of regaining function\textsuperscript{58}. The behavior of IECs is tightly controlled through locally excreted growth factors, cytokines and/or bacterial products. These factors induce signaling cascades leading to activation of master transcription factors such as NF-kB and STAT-3 in IECs. These induce anti-apoptotic and proliferative effects, leading to augmented survival and division of IECs\textsuperscript{59}

In IBD repeated intestinal epithelial damage with disruption of barrier function is a key feature, and mucosal healing has recently been established as a key prognostic parameter\textsuperscript{59}.
Inherent and acquired polarity dysfunction effects on morphogenesis and function in IECs
Dysregulation of (intestinal) epithelial morphogenesis is precursor to epithelial dysplasia, polyposis and eventually colon cancer. Cancer arises in the epithelium of the crypt, where the IECs proliferate and differentiate. Uncontrolled proliferation is one of the first steps in cancer progression, and occurs after IECs have obtained the capacity to survive while no longer in a monolayer, no longer attached to the underlying tissue. Hyperproliferation leads via an adenoma stage to a cancerous stage while molecular alterations stack (chromosomal instability, microsatellite instability, hypermethylation, early APC mutation, late p53 mutations). Inflammatory bowel diseases are associated with an enhanced risk of colorectal cancer up to three times the risk in the general population, with a longer disease duration, greater extent and severity of inflammation associated with an increasing risk. An intricate interplay between cytokines, immune cells and intestinal epithelial cells, possibly in predisposing genetic and/or environmental contexts, likely drives the development of IBD associated colorectal cancer. Inflammation in IBD leads to increased proliferation and regeneration and thus increased cell turnover with resultant increase in the number of mutations. Consequently, the events leading to eventual cancer happen faster. Histologically, IBD-associated colorectal cancer follows a different pathway, that is, from inflamed, regenerative epithelium, to hyperplastic epithelium, to flat dysplasia, and finally to invasive adenocarcinoma. Epithelial regeneration and dysplasia may be difficult to distinguish histologically. Also, as opposed to sporadic colorectal cancer development, APC mutations occur relatively late, and p53 mutations occur relatively early, and can even be found in non-dysplastic lesions. These findings have led to a theory of 'field cancerization', where a complete
area of inflammation-affected epithelium is prone to progress to a cancerous phenotype. Thus, loss of polarity and differentiation in IECs to cancer progression can be caused by a combination of intrinsic and extrinsic factors.

**Loss of polarity mechanisms leads to loss of epithelial characteristics.**
Defective epithelial barrier function frequently leads to diarrhea, and can be caused on the cellular level by defective cell polarity mechanisms. Tight junctions are the rate limiting factor for epithelial permeability in an otherwise intact monolayer. Therefore, disruption of tight junctional protein function or localization might cause impaired barrier function.

Tight junctions are dynamically endocytosed and recycled via apical recycling endosomes. Microvillus inclusion disease (MVID) is a rare disease clinically characterized by an early-onset severe secretory diarrhea, caused by polarization defects due loss of function of Myosin Vb, a key regulator of recycling endosomes, encoded by the MYO5B gene.

MVID IECs are characterized by microvillus atrophy, the intracellular retention of transmembrane apical brush border proteins, and the name-giving cytoplasmic microvillus inclusions including the main brush border anion transporter cystic fibrosis trans-membrane conductance regulator and the NHE-2 and -3 Na+/H+ exchangers and some basolateral proteins.

**IECs need a tight control of their metabolic pathways to ensure proper morphogenesis**
The two main energy metabolism pathways in mammalian cells are mitochondrial oxidative phosphorylation and (aerobic) glycolysis. During oxidative phosphorylation, adenosine triphosphate (ATP) is formed following the oxidation of nutrients.
in mitochondria. During glycolysis, glucose is converted into pyruvate thereby generating ATP. Glycolysis is relatively inefficient compared to oxidative phosphorylation, generating two ATP molecules per molecule glucose, versus 36 in oxidative phosphorylation. This, however, can be compensated for by the higher rate by which aerobic glycolysis can take place, and aerobic glycolysis allows proliferating cells to use glycolytic intermediates as macromolecular precursors facilitate biomass accumulation\textsuperscript{82}. Rapidly proliferating tissue has a relative increase in glycolysis. In the mouse intestine a relative increase in glycolysis is found in the (proliferating) crypt epithelium compared to the (differentiated) villus epithelium. A particular proliferative tissue, the epithelium-derived colon carcinoma, also undergoes this metabolic shift, and recently a mouse model professing an increase in glycolysis has been found to have an increased change of colon carcinoma development\textsuperscript{83}. Taken together, intestinal epithelial cells need to control their metabolic pathways in order to maintain homeostasis in their dynamic environment.

The need for a reductionist \textit{in vitro} model mimicking key \textit{in vivo} aspects to study intestinal epithelial integrity and morphogenesis

An intestinal disease phenotype is the product of activities of many cell types, including IECs, communities of commensal or pathogenic microbiota living in the gut and immune cells. While animal models and clinical studies are very useful to assess correlation and causation of interventions to total disease phenotypes, such approaches do not easily allow to determine the specific role of the epithelium. The epithelium, however, is the functionally impaired cell layer. Therefore, a reductionist approach is warranted for studying epithelium specific effects. By isolating the epithelium, we can assess effects of different factors
considered pathogenic or ameliorating, excluding that the epithelial effect perceived is in fact secondary to a contextual change. In this way, deeper knowledge of disease mechanisms may be obtained.

For *in vitro* assessment of epithelial morphogenesis, a model mimicking key aspects of *in vivo* morphogenesis is necessary. Cell lines that are able to obtain morphological and biochemical features of intestinal epithelial cells, such as CaCo-2 cells$^{84-89}$ that show contact-inhibition of growth and changes in the expression levels of mRNA$^{86,87,90}$ and proteins$^{84,88}$ associated with their proliferation and differentiation which are similar to those during enterocyte differentiation in vivo$^{87}$, are therefore suited best for morphogenesis studies. The recent advances in culturing primary intestinal stem cells, giving rise to organoids bring the possibility of creating a model system that might be even closer to the *in vivo* situation. However, cell lines are well characterized and isogenic. Organoids will differ from donor to donor, and indeed even from part to part in the same intestine$^{91}$. Therefore, organoids are per definition not as well characterized, making the distinction between a treatment effect or specific-organoid-intrinsic factor more difficult to make. This makes cell lines perhaps more suitable as a primary tool to decipher mechanistic features of a disease phenotype.

Three-dimensional cultures are a relatively new method in the assessment of epithelial function and morphogenesis. Certain intestinal epithelial cell lines, amongst which is CaCo-2, are capable of forming a three-dimensional structure when given the correct cues and physical surroundings. When plated in the presence of matrigel; a gel resembling extracellular matrix, CaCo-2 cells clonally proliferate and start polarizing after the first division. In a two-cell stage the IECs create an apical membrane at the cell-cell interface, and further division happen perpendicular to the apical-basal axis. As fluid is excreted from
the apical side, a lumen is created between the cells. Eventually the cell cluster thus resembles a hollow ball lined by a monolayer of IECs whose apical membranes are directed to the lumen with microvilli protruding into the lumen, and basally a basement membrane is recruited, analogous to the *in vivo* situation. Analogous to *in vivo* situation, the 3D-cultured IEC must be capable of (1) interlinking to neighbor cells, otherwise gross organization would be lost, (2) separating membrane domains, otherwise the cell could not organize itself respective to its environment or create a lumen, (3) the IEC must have functional TJ, otherwise the sphere would not be impermeable, (4) distributing cellular components in a polarized fashion, as evidenced by e.g. the microvillus localization, (5) dividing in planar orientation, otherwise a monolayer could not be created or maintained. Thus, to be able to form such a spheroid, the IECs morphogenesis in culture must resemble in vivo morphogenesis in all crucial ways (See figure 2 for a schematic overview of a spheroid). Therefore, this model is an appropriate approach for studying morphogenesis, analogous to *in vivo* epithelial (re)generation.

**Scope of this thesis**
The aim of this thesis is to investigate the direct effects of different pathogenic factors on intestinal epithelial morphogenesis and function. Previous studies have mainly focused on the assessment of either the complex interplay of all cell types in the gut, or used conventional, two-dimensional cell culture systems lacking essential characteristics of *in vivo* epithelium. In this thesis we developed and employed a reductionist model system that bridges the gap between conventional two-dimensional cell culture and *in vivo* animal models/humans to investigate the direct effects of potentially pathogenic extrinsic factors (including pro-inflammatory cytokines, changes in energy metabolism, and
cigarette smoke) as well as intrinsic factors on intestinal epithelial morphogenesis and integrity.

**Figure 2 Schematic overview of a typical intestinal epithelial spheroid**, showing an established lumen, faced by apical membranes delineated by tight junctions. On the left a see-into 3D model is shown, on the right a cross-section of the sphere on the left is shown.

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Introduction & scope of the thesis


Introduction & scope of the thesis


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