Chapter 7

Summary and future perspectives

Leon J. Klunder

Department of Cell Biology, University Medical Center Groningen, University of Groningen, Groningen, the Netherlands
Summary

The intestinal epithelium is capable of taking up nutrients and fluid, and forming an otherwise impermeable layer. In order to do this, intestinal epithelial morphogenesis is tightly regulated, giving rise to an interlinked, polarized monolayer of intestinal epithelial cells. Cell polarity is pivotal for epithelial homeostasis and immunity in the intestine, as discussed in detail in Chapter 2. Under influence of intrinsic and extrinsic factors, morphogenesis, and thereby the function of the epithelium can be perturbed. Animal models and clinical studies provide information on disease phenotypes in the context of complex interplay of many different cell types. In this thesis we have presented a three-dimensional cell culture system, reflecting the in vivo polarized organization of the intestinal epithelium, as a reductionist approach to investigate the direct effects of individual extrinsic and intrinsic factors on intestinal epithelial morphogenesis and integrity.

In chapter 3 we thus demonstrated that intestinal epithelial cells form a epithelial spheroid consisting of a cell monolayer surrounding a central lumen, reflecting the in vivo glandular organization of intestinal epithelial tissue. In this spheroid, the intestinal epithelial cells show typical in vivo-like polarized distributions of cellular components. The intestinal epithelial cells display distinct apical and basolateral membrane domains, with microvilli projecting into the lumen, and the basement membrane component laminin being recruited at the cell-ECM-interface. The tight junctions are properly localized and functional, as evidenced by the impermeability of the spheroids. We demonstrated that exposure of pre-formed spheroids to TNFα enhanced the permeability of the spheroids in an apoptosis-dependent manner. Further, the exposure of intestinal epithelial cells to TNFα inhibited the initial development of an apical-basal polarity axis, and lumens formed only secondarily, after the inner cells of
a cluster became apoptotic, a process called cavitation and typical for mammary gland morphogenesis. This demonstrated that TNFα not only exerted an apoptosis-dependent perturbing effect on intestinal epithelial barrier function, but also a perturbing effect on intestinal epithelial morphogenesis.

In chapter 4 we assessed the role of energy metabolism pathways in intestinal epithelial morphogenesis and the underlying processes of cell polarization and proliferation in our 3D model system. We demonstrated that two main metabolic pathways, glycolysis and oxidative phosphorylation, both contribute to proliferation and spheroid size. Neither pathway however, was individually necessary for efficient intestinal epithelial cell polarization and the development of glandular spheroids architecture. We demonstrated that, in contrast to TNFα (chapter 3), IFNγ significantly enhanced intestinal epithelial cell proliferation in this model system, resulting in spheroids of larger size. This stimulation of proliferation was shown to require glycolysis but not oxidative phosphorylation. This study for the first time addressed the role of energy metabolisms in 3D intestinal epithelial morphogenesis, and demonstrated that cell proliferation and polarization, both key to epithelial morphogenesis, differently utilized energy metabolism pathways. Importantly, the studies described in chapters 3 and 4 demonstrate that different pro-inflammatory cytokines, i.e., IFNγ (chapter 4) and TNFα (chapter 3), affected intestinal epithelial morphogenesis in this 3D model in very different manners.

In chapter 5 we employed the 3D intestinal epithelial model system to investigate the effects of cigarette smoking as a IBD risk factor on epithelial barrier function and morphogenesis. We demonstrated that exposure of intestinal epithelial cells to cigarette smoke extract resulted in impaired proliferation, cell polarization, resulting in defective epithelial
morphogenesis. Moreover, these effects of cigarette smoke extract were transferrable to daughter cells. Interestingly, cigarette smoke extract did not perturb the barrier function or morphogenesis of pre-formed intestinal epithelial spheroids, indicating that the effects of cigarette smoking on intestinal epithelium may be particularly relevant during active morphogenesis such as during the wound healing response. Finally, we demonstrated that the administration of N-acetyl-L-cysteine (NAC) completely neutralized the inhibitory effects of cigarette smoke extract via glutathione dependent and –independent manners. This study demonstrated that cigarette smoke components can exert direct effects on intestinal epithelial cells, and that NAC may be used to neutralize cigarette smoking-mediated intestinal epithelial defects.

In chapter 6 we assessed the influence of apical recycling endosome regulator myosin Vb on intestinal epithelial barrier function. We demonstrated that loss of Myosin Vb function can be causally linked to the redistribution of Claudin-1 in enterocytes of individuals with microvillus inclusion disease. We demonstrated that Myosin Vb was necessary to regulate the trafficking of Claudin-1 to the tight junctions, but did not control the correct localization of other TJ proteins. The TJs were functional, given that permeability was not altered in the absence of myosin5b and correctly localized Claudin-1. These results indicated that Claudin-1 mislocalization as such is not likely to be causative factor in disorders associated with increased intestinal permeability. In intestinal epithelial cells of individuals with microvillus inclusion disease carrying MYO5b mutations, we found a loss of Crumbs3 expression, and in Crb3−/− mice we found a mislocalization of Claudin-1. Thus, Myosin Vb controlled Claudin-1 trafficking is associated with the proper expression of Crumbs3. Importantly, the lack of intestinal epithelial barrier defects in the absence of myosin Vb indicated that intestinal epithelial
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permeability is not likely the cause of the diarrhoea phenotype observed in MVID.

Future perspectives
In this thesis we established an *in vitro* three-dimensional cell culture model system for the study of intestinal epithelial morphogenesis. In this model system, single intestinal epithelial cells self-organize to form a multicellular polarized architecture that best reflects the physiological *in vivo* situation and therefore provide a suitable approach bridging the gap between conventional cell culture and *in vivo* animal models/humans. We have applied this model to investigate the direct effects of extrinsic factors on intestinal epithelial morphogenesis and integrity. We provide a first description of the perturbation of morphogenesis caused by different pathogenic factors, including pro-inflammatory cytokines, changes in energy metabolism, and cigarette smoke. The next step would be to further elucidate the molecular mechanisms behind the different morphogenetic and functional perturbations as described in this thesis.

The effects of TNFα, altering intestinal epithelial morphogenesis to a cavitation-like phenotype, raise the question of whether the mechanisms behind the secondary lumen formation are mechanistically comparable to other tissues with similar epithelial lumen formation process, such as mammary and slavery gland epithelium 1-3. The pro-proliferative effects of IFNγ warrant further mechanistic research. Given that IFNγ can cause TJ mislocalization, a possible mechanism, postulated in chapter 4, could include the nuclear translocation of proliferation-stimulating transcription factors that are in normal conditions physiologically sequestered at functional TJs4,5. The studies described in this thesis demonstrated that TNF and IFNγ directly, but differentially, influence intestinal epithelial morphogenesis. Considering the heterogeneity of
the disease course in IBD, including the notion that not all patients eventually develop colon cancer\textsuperscript{6-8}, it is intriguing to consider the possibility of different cytokine profiles giving rise to different disease phenotypes and disease course. The perturbation of intestinal epithelial morphogenesis by cigarette smoke extract exposure is likely caused by an effect in early cell polarization. The penetration of the effects of cigarette smoke extract into next generations of cells warrants further investigation into the potential effects of cigarette smoke exposure on genetics and/or epigenetics changes, and their potential contribution to the observed cell polarity and morphogenesis defects. Recently developed methods to culture intestinal stem cell-derived organoids provide the option of a disease or location-specific readout of effects on (regenerative) morphogenesis\textsuperscript{9}. Organoids from inflamed and healthy IBD tissue might be compared to non-IBD-tissue organoids in their respective reaction to different (inflammatory) stimuli. In this way the possibility of inherent differences in epithelium from the healthy population as a cofactor in causing IBD could be investigated. Another option would be to discern whether the differential effect of cigarette smoke in Crohn’s disease and ulcerative colitis is due to a difference in direct smoke-epithelial interaction. Also, given our results showing that the effects of cigarette smoke are transferable to next generation daughter cells, it might be interesting to assess the morphogenesis capacity of intestinal organoids from cigarette smoking healthy and IBD donors. A further step in research would be the expansion of the current reductionist three-dimensional intestinal epithelial cell model to include other cell types involved in disease pathogenesis. As IBD is a multifactorial disease, with many cell types which in context create a disease phenotype, a stepwise expansion would provide the greatest insights,
eventually. In this way, the role of every cell type and its functional relation to epithelial morphogenesis can be assessed. For example, co-culturing intestinal epithelial cells with immune cells or the microbiota samples from patients suffering from IBD might give valuable insight in any of these factors interfering with epithelial morphogenesis. This would further validate the possible specificity of effects on epithelium as described in this thesis.

As spheroid or organoid formation resembles *in vivo* epithelial (re)generation, organoid morphogenesis might provide a readout for regenerative capacity of a specific donor or patient. We might then co-culture the patient’s own intestinal epithelial cells, with his/her own immune cells, and start recreate the immunological microenvironment. This would provide the opportunity to experimentally, *in vitro*, determine whether a treatment might help epithelial healing *in vivo* and, thereby, improve disease course, analogous to the use of the recently developed tumor organoids\(^{10,11}\).

As we propose that our model system is an *in vitro* analogy to *in vivo* intestinal epithelium (re)generation, we ultimately need an *in vivo* system to further substantiate this claim, where the proposed morphogenetic effects can be tested. However, to follow morphogenesis in time *in vivo* a method utilizing dynamic imaging would be necessary. An option would be creating mice with GFP attached to different polarity markers, and assessing morphogenetic perturbations in epithelial (re)genesis in time, by operatively taking out the intact intestine, and peri-operatively using fluorescence microscopy, or using fluorescence endoscopy.

In conclusion, model systems that make it possible to study epithelial morphogenesis as a factor in diseases that impair epithelial homeostasis and immunity in the gut provide promising tools for deeper understanding of disease pathogenesis.
References


