Intestinal Organoids – a Powerful Model to Study Interactions of the Epithelial Barrier with its Environment

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Abstract
The gastrointestinal (GI) tract is the body’s largest mucosal area exposed to the external environment. It harbours a large and diverse community of commensal microorganisms. The GI tract is lined by a monolayer of epithelial cells that separates the intestinal lumen and underlying tissues. It is not only a physical barrier - it interacts with commensal microorganisms and immune cells by mechanisms that are not fully defined. The intestinal immune system must maintain the balance between offering adequate protection against pathogens, while remaining tolerant to harmless antigens from commensals and diet. Imbalances in these immune responses may lead to the development of chronic inflammatory disorders such as inflammatory bowel disease (IBD). Despite intense research, the molecular mechanisms governing intestinal homeostasis remain to be fully understood. To help defining these complex mechanisms, we adapted the small intestinal organoid model. We isolated small intestinal crypts according to the protocol recently published by Sato and Clevers, 2013. In the presence of the growth factors epidermal growth factor (EGF), Noggin and R-spondin-1 crypts, containing intestinal epithelial stem cells, form organoids. The organoids resemble the in vivo organisation of the epithelial barrier. Technologies like confocal microscopy and live cell imaging will enable us to follow changes in the intestinal epithelial cells (IECs) in detail and in real-time. We propose that analysis of intestinal organoids in the presence of immune cells and mediators, diet-derived molecules and / or commensal microorganisms will facilitate the discovery of molecular mechanisms directing intestinal homeostasis, thus making it a powerful model to study the interactions of the epithelial barrier with its environment.

Keywords: Intestinal organoid, intestinal epithelial cells (IECs), intraepithelial lymphocytes (IELs), mucosal immunology, intestinal homeostasis

Introduction
The immune system consists of humoral-mediated and cell–mediated immune responses, which collectively protect the host from the risk of infections by bacteria, viruses and parasites. This task is particularly challenging at epithelial barrier sites,
such as the skin and the GI tract, as they are directly exposed to the external environment. In addition, they harbour commensal microorganisms. There are approximately $10^{14}$ commensals in the intestine (Artis, 2008), which live in harmony with their host. However, there is a constant risk that some become opportunistic pathogens or are displaced by pathogenic microorganisms. This is most apparent in the largest mucosal area exposed to the external environment, the GI tract, which is constantly exposed to antigens from the diet and the commensal microorganisms. Thus the intestinal immune system must maintain the delicate balance between protective and tolerant immune responses. Failure in remaining this balance may result in intestinal disorders such as inflammatory bowel disease (IBD) (Artis, 2008).

An essential player in intestinal homeostasis is the epithelial barrier, which is the first line of defence against pathogens. The epithelial cells of the small intestine are organised into villi and crypt structures; Villi are epithelial projections into the intestinal lumen, while the crypt structures are epithelial invaginations (as illustrated in figure 1 in Moens and Veldhoen, 2012). Intestinal epithelial stem cells, which have the potential to generate all subsets of epithelial cells, are situated near the base of the crypt. Intestinal stem cells generate daughter cells called transit-amplifying (TA) cells, which either return to stemness or differentiate into a secretory epithelial cell lineage (Paneth, goblet, enteroendocrine cells and tuft cells) or into enterocytes. Paneth cells reside within the crypt structure and secrete various antimicrobial proteins (AMPs) such as cryptdins and lysozyme. Other epithelial subsets migrate up the crypt-villus axis, like goblet and enteroendocrine cells, which secrete mucus and various hormones, respectively. Tuft cells received their name from their apical tufts of microvilli and are thought to participate in the chemical sensation of the luminal contents (Clevers, 2013) The major cellular subset of the epithelium are enterocytes, which absorb fluids and nutrients from our diet. They account for approximately 80 % of all cells of the epithelial barrier (Clevers, 2013; Moens and Veldhoen, 2012; Peterson and Artis, 2014).

The epithelial barrier and the overlying layer of mucus and effector molecules form a physical barrier, but the epithelial cells do interact with commensal microorganisms at the apical side. At the basolateral side, the IECs interact with cells and mediators of the immune system. For example, intraepithelial lymphocytes (IELs) reside in close proximity of the epithelium. On average, there is one IEL for every 4-10 IECs in the mouse small intestine (Edelblum et al., 2012). However, the molecular mechanisms involved in the interactions between IECs and IELs are poorly defined. Dysregulation of this finely tuned system may contribute to development of inflammatory diseases such as food allergies, celiac disease, inflammatory bowel disease (IBD) and intestinal cancer (Karin et al., 2006; Macdonald and Monteleone, 2005).

Defining the complex interactions between the epithelial barrier and its environment in vivo is difficult and requires the use of animal models. In addition, intravital
imaging in mice is a highly complex methodology, requiring specialist skills. Recently, Ritsma et al. published a protocol of live mouse imaging using an abdominal imaging window technology (Ritsma et al., 2013). However, the method requires both invasive surgery and fluorescent reporter mice for imaging of particular cell subsets.

There are cell culture systems, such as the intestinal epithelial cell line Caco2, that have been used to study interactions between IELs and IECs (Edelblum et al., 2012). However, cell lines cannot recapitulate the complex spatial (3D) organisation of the intestinal epithelium. Furthermore, cell lines have undergone significant molecular changes to become immortal and do not represent all intestinal subsets - hence do not represent the in vivo situation accurately. These issues were overcome by the intestinal sphere culture system. The system took advantage of intestinal stem cells in an air-liquid interface system, supplemented with underlying stromal elements, for long-term cultures. However, the intestinal spheres culture failed to recapitulate the characteristic villus-crypt morphology (Ootani et al., 2009).

A recent in vitro model that does recapitulate the in vivo organisation is the intestinal organoid culture system (Sato and Clevers, 2013). This method takes advantage from the presence of intestinal stem cells in the crypts and makes use of a mixture of extracellular matrix proteins (Matrigel) that allows three-dimensional growth. This system closely resembles the in vivo tissue organisation.

The organoid model will enable interactions studies between the epithelial barrier, microbiota and immune cells in detail and real time. The interactions can be assessed in both homeostatic and inflammatory conditions. We have successfully set up and adapted the organoid culture system in our lab.

**Material and methods**

**Animals**

C57BL/6J mice were maintained at the Babraham Institute in accordance with the Babraham Institute Animal Welfare, Experimentation & Ethics Committee and the UK Home Office.

**Small intestinal organoid setup and maintenance**

The proximal part of the small intestine was cut longitudinally, washed in PBS (Ca$^{2+}$/ Mg$^{2+}$ - free) and cut into 2-4 mm pieces. The pieces were washed 10 times in PBS prior to chelation in PBS / 2mM EDTA at 4 °C for 30 min. Afterwards the intestinal pieces were mixed in PBS several times before the supernatant (subsequently named fraction) was collected and replaced with new PBS. Crypt-enriched fractions were identified, filtered (70μm) and washed twice in basal culture medium (advanced DMEM/F12, 2 mM GlutaMax, 10 mM Hepes, , supplemented with 100 U/ml penicillin/ 100 μg/ml streptomycin all from Life Technologies).The crypts were resuspended in
Matrigel (356231, BD Bioscience) and hemispherical droplets (50 μl) were formed in pre-warmed 24-well plates. After polymerisation of the droplets for 10 min at 37°C and 7 % CO₂, 500 μl complete crypt culture medium (basal culture medium supplemented with N2 (1X), B27 (1X), 50 ng/ml EGF, all from Life Technologies, 1 mM N-acetylcysteine (Sigma Aldrich), 100 ng/ml Noggin (ebioscience), 1 μg/ml R-spondin-1 (R&D systems)) were added per well. The crypts were maintained in 37°C, 7 % CO₂. The complete crypt culture medium was exchanged 4 days after culture setup. After 7 days the cells were split by mechanical disruption of the organoids by successive use of a p1000, p200 and fire-polished Pasteur pipette. The cells were then washed twice in basal culture medium prior to re-plating, as described above.

**Imaging of intestinal organoid**

Bright-field images were acquired by a Zeiss Axio observer microscope. Image analysis was performed using Zeiss AxioVision (v.4.8.3) and ImageJ (v.1.47V). For fluorescence images the organoids were stained with Hoechst (2 mg/ml) and CellMask (5 μg/ml), both from Life Technologies, diluted in basal culture medium at 37 °C for 10 min. An Olympus FV1000 confocal microscopy system was used for imaging and Imaris software (Bitplane) for image processing.

**Results**

To establish and optimise the intestinal organoid culture in our lab, we adapted the published protocol (Sato and Clevers, 2013) by changing one parameter at a time.

First, we addressed whether less mechanical stress during crypt isolation would improve the success rate of organoid culture. This was done by pipetting the intestinal fragments more gently during the washing steps (data not shown). When the more gently pipetting still generated villi-enriched fractions, the less gentle pipetting generated crypt-enriched fractions. This led to a better separation of villi- and crypt-enriched fractions, thus providing us with a purer crypt suspension for the start of the organoid culture.
Figure 1. Example of fractions for set-up of organoid cultures. (A) The two first fractions contain mainly larger intestinal pieces and villi structures. The fractions become enriched in crypt structures (seen as the round structures in the figures) from fraction number 3 with decreased number of crypts for each of the following fractions. (B) The black arrows point at what we would define as crypts, while the red arrows point that those that could both be crypts or fragments of a villi.

Subsequently, we assessed whether the quantity of crypts in a fraction or their purity was most critical for organoid yield. Following chemical (2mM EDTA) and mechanical disruption of the intestinal epithelial layer, fractions were inspected for their content. Fraction number 3 was most crypt-enriched, with less crypts being present in the following fractions (Figure 1). We set up cultures from pooled fractions 3-5, 4-6 and 6-8. Both fractions 3-5 and 4-6 successfully generated organoid cultures, whereas none of the fraction 6-8 cultures have achieved this, despite being more enriched for crypt structures (Figure 1). These data suggest that the quantity of crypts is more critical for successful organoid culture than the purity of the fraction.

We then tested various crypt concentrations: 200-500, 1000, 2000 and 4000 crypts per 50 µl Matrigel droplet. Of note, due to the difficulty to accurately distinguish crypt structures from some villi fragments, the counted number of crypts may vary from the actual amount present in the droplet. Especially apical portions of the villi have a similar morphology to crypts, as illustrated in figure 1B. Both 1000 and 2000 crypts per 50 µl matrigel droplet successfully formed organoids (data not shown).

To summarise, neither increased crypt density nor additional crypt fraction purification did improve the success rate of the crypt culture set-up.
Once the crypts had been plated, selected crypts (organoids) per well were followed on a daily basis using bright-field microscopy. 48 h after culture set-up, we observed that the organoids had expanded in size and started to bud (Figure 2B). These buds represent de novo crypt structures. The number of buds varies among individual organoids (Figure 2A+B+D). At day 5 we observed accumulation of shed epithelial cells in the organoid lumen, which is likely to be a result of epithelial turnover, which approximately takes 5 days (Sato and Clevers, 2013). Notably, organoid growth was not synchronised within a well, as we observed organoids with different numbers of buds, or even without any (Figure 2C). Further optimisation might be able to solve this issue.

Figure 2. From a single crypt to an organoid. (A) Intestinal organoid with 6 crypt structures (buds) 5 days after culture set-up. (B) Time lapse live cell imaging of organoids. The black arrows show the fast formation of a bud in the organoid. (C) The growth kinetics varies in the same Matrigel droplet. These organoids have been in culture for 4 days.

To test the staining procedure on organoids and to investigate the organoid structure in 3D, we performed a fluorescence staining using Hoechst and CellMask, to stain nuclei and cell membranes, respectively. The imaging showed an intact organoid structure with crypt and villi domains, as published previously (Figure 3 and (Sato and Clevers, 2013). Thus, the system is set up for analysis by widefield fluorescence and confocal microscopy.
Figure 3. Confocal imaging of a 5 day old organoid. The organoid was stained with CellMask (cell membrane) and Hoechst (cell nuclei) to illustrate the 3D structure of the organoid.

Discussion
We successfully adapted the culture of small intestinal organoids in our lab. However, there are still several parameters that require optimisation.

Interestingly, it seems that, at the start of the culture, the quantity of crypts is more important than the purity of the fraction. Despite contamination of villi fragments that will undergo apoptosis within a few days, crypts manage to form organoids. The dead epithelial cells will be washed out during passaging of organoids. After the first passage, organoids can be used for further experiments such as co-culture experiments, whereas the optimal time point for the start of the co-culture remains to be determined empirically.

Further method optimisation would be required. For example, we observed great variation in organoid formation between individual organoids in the same culture well. This can be assessed by titration of the different growth factors. It is possible that one or more of the growth factors would need to be in present at higher concentration to obtain more uniform organoid growth. A more uniform organoid formation would make the model more sensitive for detecting smaller changes in epithelial growth and cell differentiation.
Furthermore, we have also performed fluorescence imaging of the organoids with promising results, as illustrated in figure 3. In future experiments we will expand the staining panel with antibodies identifying different epithelial subsets. This approach will illustrate the epithelial organisation, which we can use to compare to organoids cultured in the presence of immune cells, immune mediators and pathogens or their components to detect differences.

In addition, others have successfully performed techniques such as retroviral transduction, RNA interference (RNAi) and bacterial artificial chromosome (BAC) technology on the organoid system (Sato and Clevers, 2013; Schwank et al., 2013). Furthermore, the source of the organoid can originate from various knock-out or knock-in mice strains that are engineered to have fluorescent reporters, making the intestinal organoid system a powerful model.

The intestinal organoid system is currently used in various research fields. The model has used it to characterise important factors for intestinal stem cells and their differentiation. The organoids could also be used for intestinal cancer research (Sato and Clevers, 2013). It is also has potential as drug candidate testing model (Astashkina and Grainger, 2014). We will add intestinal immunology interactions research to the list of the organoids’ potential.

We will co-culture intestinal organoids in the presence of IELs and compare the co-cultures with organoids only cultures. Previous data has suggested that IELs increase the turn-over rate of IECs (Boismenu and Havran, 1994; Hidaka et al., 2012; Li et al., 2011). The IELs is a heterogeneous pool of T cells that can be divided into three main groups; the conventional IELs that express the classical αβ T cell receptor (TCR) with either CD4 or CD8αβ as co-receptors, the unconventional IELs expressing CD8αα and the IELs expressing the γδ TCR and no co-receptor. (Guy-Grand et al., 2013). Only the γδ IELs have been reported to secrete the epithelial cell mitogen keratinocyte growth factor (KGF), thought to be involved in the IECs turn-over (Boismenu and Havran, 1994). However, the other IELs groups could also influence the epithelial cell turn-over by mechanisms that are unknown at the moment. Whether the IELs effect on turn-over rate affects all the epithelial cell subsets or only some specific ones remain to be discovered.

Another interesting question to address is whether the IELs affect IECs with respect of cytokine, AMPs and hormone production. Any changes in the amount of the cytokines, AMPs and hormones will probably affect the possibilities the epithelial cells have to maintain the intestinal homeostasis in both steady state and during inflammation and infection. It has been reported that the IECs affect the production of IFNγ among CD4+ IELs (Hatano et al., 2013).

IELs do not only interact with the IECs, but also with the diet. In mice that lack the nuclear receptor aryl hydrocarbon receptor (AhR), whose ligands are mainly diet-
derived, the IELs were absent in the intestinal compartment. The AhR knock-out mice had significant increased bacterial load in both the small intestine and the colon. Furthermore, the AhR knock-out mice had increased severity of colitis compared to control mice (Li et al., 2011). These results indicate the importance of the interactions occurring among the diet, IECs and IELs to remain intestinal homeostasis. However, their molecular mechanisms remain to be further characterized.

Similar experimental approaches can be used to address the molecular mechanisms governing microbiota-epithelial interactions. The organoids could be infected with intracellular pathogens. Recently, Wilson et al. used the organoid system to investigate the mechanisms behind Salmonella enterica infection. The intestinal lumen in the organoids could be assessed by microinjections with the bacteria and still remained intact for studies (Wilson et al., 2014).

In summary, intestinal organoids are a powerful model to study the interactions of the epithelial barrier with its environment, namely the commensal microorganisms, the immune system and dietary components.

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References


