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Novel approaches: Tissue engineering and stem cells – *In vitro* modelling of the gut

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A B S T R A C T

In many intestinal diseases, the function of the epithelial lining is impaired. In this review, we describe the recent developments of *in vitro* intestinal stem cell cultures. When these stem cells are grown in 3D structures (organoids), they provide a model of the intestinal epithelium, which is closely similar to the growth and development of the *in vivo* gut. This model provides a new tool to study various diseases of malabsorption in functional detail and therapeutic applications, which could not be achieved with traditional cell lines. First, we describe the organization and function of the healthy small intestinal epithelium. Then, we discuss the establishment of organoid cultures and how these structures represent the healthy epithelium. Finally, we discuss organoid cultures as a tool for studying intrinsic properties of the epithelium, as a model for intestinal disease, and as a possible source for stem cell transplantations.

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The intestinal epithelium

The small intestine is with approximately six meters the longest organ in our body. It has a tube shape and consists of different tissue layers: the serosa and the muscularis externa on the outside, the submucosa (stromal layer) in the middle, and the mucosa in the inside of the intestinal tube. The mucosa is composed of the muscularis mucosae (smooth muscle layer), the lamina propria (connective tissue), and a single layer of epithelial cells facing the lumen of the intestine. This epithelial sheet is responsible for digestion and nutrient uptake. The epithelium is highly organized and contains protrusions on several levels (called villi and microvilli), which allow for a tremendous increase of the surface. If flattened out, the epithelium of an adult person could cover ~30 m^2 area [1]. In many intestinal diseases (enteropathies), the function of this epithelial lining is impaired [2].

The epithelial cell layer in the small intestine is organized into proliferative compartments and differentiated compartments. The proliferative compartments, which also contain the stem cells, are known as the crypts of Lieberkühn, and are embedded in the submucosa [3]. Several crypts surround and fuel one differentiated compartment, known as a villus [4,5]. The villi protrude into the lumen of the small intestine.

The organization of the epithelium is the same throughout the whole small intestine, however the morphology varies a bit: the most proximal part of the small intestine - the duodenum - shows a high abundance of very long villi, whereas the number and lengths of the villi decrease towards the jejunum (the middle part) and the ileum (the most distal part of the small intestine) [6]. The colonic epithelium consists of a flat differentiated compartment rather than villi.

Intestinal epithelial cell types and homeostasis

The intestinal epithelium is constantly regenerating, with an enormous cellular turnover. New cells are generated in the crypts. They proliferate in the crypts, migrate and differentiate upwards on the villi and undergo anoikis (cell-detachment-induced apoptosis) at the tip of the villi, and are then shed into the lumen of the small intestine [7]. In humans, approximately 100 billion cells are estimated to be replaced every day [8].

The intestinal stem cells

The self-renewing capacity of the intestinal epithelium depends on the presence of intestinal stem cells. Multipotent adult intestinal stem cells are defined as cells that give rise to all differentiated intestinal epithelial cell types, and are at the same time capable of self-renewal. Actively cycling intestinal stem cells are located at the bottom of the crypts. Those cells were first mentioned in 1887, in a publication by Josef Paneth, who termed them “schmale Zellen” (=slender cells) [9]. In 1974, Cheng and Leblond characterized those cells as *crypt base columnar* cells (CBC cells), and proposed them as intestinal stem cells [10,11]. In 2007, lineage-tracing experiments showed that CBC cells are actively cycling, undergo self-renewal, and generate all mature intestinal epithelial cell types, proving they are multipotent stem cells [12]. The CBC stem cells express several unique marker genes, such as Lgr5 and olfactomedin-4. Upon injury and stem cell loss, cells from a quiescent stem cell pool higher up in the crypt (so-called +4 stem cells or label retaining cells), and secretory progenitor cells can dedifferentiate to replace the pool of active cycling Lgr5-positive CBC stem cells [13–17]. The CBC stem cells symmetrically divide once every 24 hours and produce new stem cells and daughter cells in a stochastic manner [18,19]. The daughter cells are termed transit amplifying (TA) cells, are highly proliferative and line the flanks of the crypts. They migrate, differentiate and give rise to the six differentiated cell types of the small intestinal epithelium.

Differentiated intestinal cells

The differentiated cell types of the small intestinal epithelium each have a specialized function in nutrient digestion and absorption, immune response or as supportive niche cells.

The absorptive **enterocytes** are the most prominent cell type in the small intestinal epithelium. Their main function is nutrient absorption, which is mediated by transporters, channels and enzymes on the plasma membranes. The apical plasma membrane of the enterocytes (facing the intestinal lumen) contains densely packed protrusions, called microvilli. This leads to an increase of the apical surface, thereby facilitating optimal nutrient digestion and absorption [20].

The mucus-secreting **goblet cells** have a goblet (cup-like) shape due to the numerous mucin granules in their cytoplasm. The secreted mucus covers the epithelium and thereby exhibits several functions. The mucus layer protects intestinal epithelial cells from physical and mechanical stress, forms a line of innate immune defense, and provides lubrication for the passage of stool [21,22].

Paneth cells reside at the bottom of the crypts, in close contact with the stem cells. They live up to three weeks, contain big granules and have two main functions. First, they produce and release antimicrobial proteins such as α-defensins and lysozyme, thereby regulating the microbiota and ensuring that the crypt remains free of microbes [23,24]. Second, they constitute the niche for the stem cells, and secrete several signalling proteins, such as WNT, epidermal growth factor (EGF) and Notch ligands [25,26].

Enteroendocrine cells are scattered throughout the villi and make up about 1% of the mucosal cells. They can be classified into different subtypes, based on the secretion of specific hormones, such as somatostatin, serotonin, or substance P, among others [27]. Enteroendocrine cells include more than ten different cell types, of which the most common are L and K cells, producing glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), respectively [28].

Tuft cells display thick tufts of microvilli on the apical surface, which might mediate the chemical sensation of luminal contents. They also produce opioids, however their exact function is still poorly understood [29–31].

M-cells (microfold cells) are a special intestinal epithelial cell type, the only one not being localized in the crypts or the villi. Instead, they reside in the follicle-associated epithelium (FAE) of the intestinal Peyer's Patches. Their main function is antigen uptake and transcytosis towards the underlying immune cells, thereby regulating immune responses [32,33].

Recently, it was demonstrated that single cell sequencing protocols are able to distinguish rare cell types in the intestine, which may reveal other unknown subsets by further detailed analysis [34].

Specification towards either the secretory lineage (goblet cells, enteroendocrine cells, tuft cells, Paneth cells) or the absorptive lineage (enterocytes) occurs in the TA lineage and is mediated by the selective activation or repression of transcription factors [35]. An overview of intestinal organization and cell types is given in Fig. 1.

Regulation of stemness, proliferation and differentiation

In order to maintain this tissue homeostasis, several cellular processes need to be coordinated, including cell proliferation, migration, differentiation and finally apoptosis (programmed cell death) [7]. Several regulatory pathways play an important role in this tight balance:

Canonical **Wnt signaling** plays a crucial role in the maintenance of stemness, the regulation of proliferation, and the differentiation of Paneth cells [26,36–38]. Wnt ligands (being secreted by Paneth cells and mesenchymal cells *in vivo* [26]) bind to cell surface receptors, such as Frizzled and LRP5/6, and thereby initiate a complex intracellular signaling cascade. This ultimately results in the accumulation of free β-catenin in the cytoplasm and its translocation to the nucleus [39]. In the nucleus, β-catenin associates with members of the TCF family of transcription factors, thereby activating a transcriptional program that maintains stem cells in their undifferentiated state [36]. Upon binding of the ligand R-spondin to its cell surface receptors Lgr4/5, this signaling cascade is further amplified [40,41].

Epidermal growth factor (EGF) signaling maintains stemness and induces proliferation. Paneth cells secrete the EGF ligand. Upon binding to its receptor (EGFR), downstream protein kinases are activated to initiate several signal transduction pathways, such as MAPK, AKT and JNK pathways, leading to cell migration, adhesion and proliferation [42,43].

Notch signaling is also involved in the maintenance of stemness and it plays a decisive role in differentiation fates. When Notch-signaling is active, progenitor cells adopt an absorptive fate and

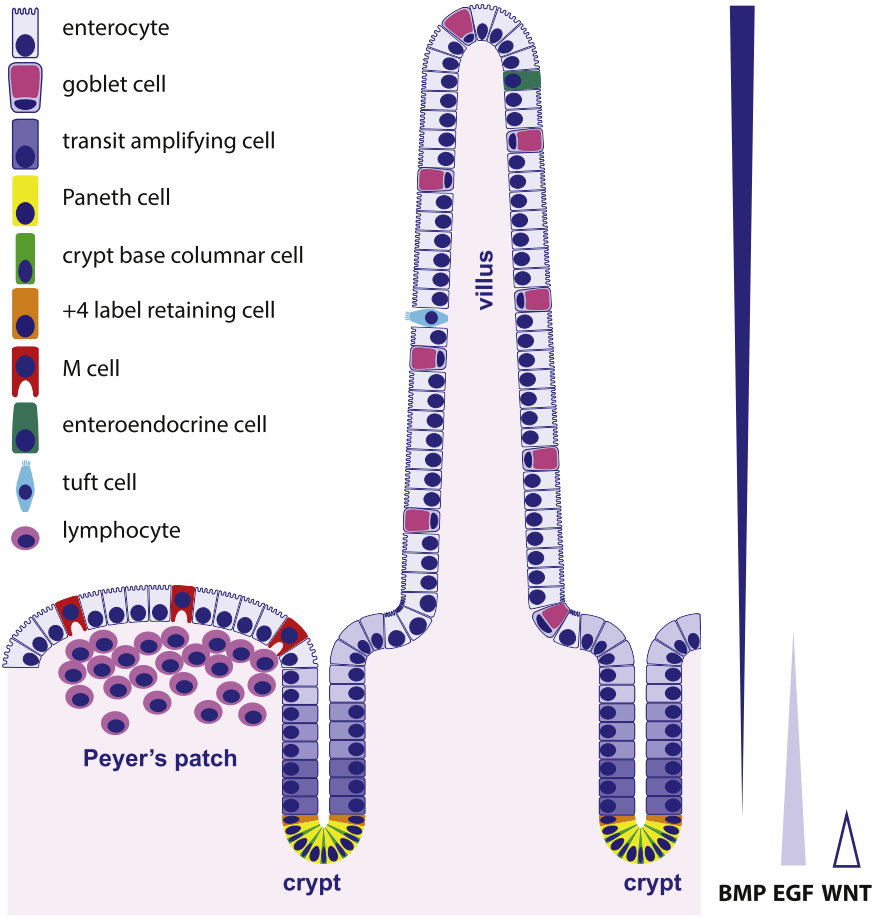

Fig. 1. Organization and cell types of the small intestinal epithelium. Schematic drawing of intestinal epithelial organization. The proliferative crypts harbor Paneth cells, intestinal stem cells, and their direct daughters, the transit amplifying (TA) cells. TA cells migrate upwards and undergo migration-associated differentiation into enterocytes, goblet cells, enteroendocrine cells or tuft cells. At the tip of the villus, cells undergo anoikis and are shed into the intestinal lumen. BMP, EGF and WNT are factors that maintain epithelial homeostasis.

become enterocytes. In contrast, when Notch signaling is switched off, cells differentiate towards the secretory lineage and become goblet cells, enteroendocrine cells, tuft cells or Paneth cells [44,45].

Bone morphogenetic protein (BMP) signaling negatively regulates stemness and is the main pathway driving differentiation of progenitor cells while migrating upwards to the villi. Accordingly, BMP signaling is only active in the villus compartment, and not in the crypts [46,47].

Intestinal epithelial polarization

In addition to this tightly regulated tissue organization, each cell itself is also highly organized. It has three membrane domains, one facing the intestinal lumen (the apical plasma membrane), one facing the neighbouring cell (the lateral domain), and one facing the underlying tissue (the basal domain). Establishment and maintenance of those distinct domains with specialized functions are crucial for proper cell functioning. If polarization is disturbed, intestinal diseases such as microvillus inclusion disease (MVID) can occur as a consequence.

Intestinal organoids

Recently, an epithelial culture system has been developed, which mimics the architecture and homeostasis of the intestinal epithelium *in vitro* [48]. These organoids self-organize into a three-dimensional "mini-intestine" and contain all intestinal epithelial cell types that are found *in vivo*, and can be expanded for years without genetic instabilities [43,49]. As such, they defeat the Hayflick limit, which states that somatic cells have a limited proliferative potential *in vitro* [50].

Organoids are cultured in matrigel to provide them with an extracellular matrix consisting of collagens and laminins that resemble the basement membrane *in vivo*. The extracellular matrix helps the epithelial cells to maintain their apical to basal cell polarity, both in matrigel (3D culture) or when cultured on transwells (2D culture) [51,52]. The media covering the matrigel consist of several growth factors, that are deduced from the *in vivo* regulatory factors that maintain stemness as discussed above. Human organoid cultures need additional growth factors and inhibitors compared to mouse cultures (Table 1). Overall, organoids almost completely reflect the *in vivo* intestinal epithelium.

Mouse organoids

Mouse intestinal organoids can be established from isolated crypts, or from single Lgr5-positive stem cells [49]. They form a central lumen that is flanked by a differentiated villus-like epithelium, and crypt-like budding structures extrude from the cystic lumen. These extrusions harbour the small intestinal stem cells, intermingled between Paneth cells at the bottom, and a TA compartment higher up. From there, cells migrate towards the villus-like compartments while differentiating, and are finally shed into the lumen of the organoids. As such, the hierarchy and homeostasis of organoids highly resembles the *in vivo* situation. Mouse intestinal organoids are grown in matrigel, and require the growth factors EGF, noggin and R-spondin in the medium [49].

By adding various recombinant proteins or chemical compounds, stem cells can be differentiated towards a specific fate (Table 1). For example, differentiation into purely enterocytes is achieved by adding extra compounds, such as valproic acid (VPA) and Wnt-inhibitor IWP-2 to the medium [53]. And incubation with RANKL will induce differentiation into M-cells, a cell-type that is normally only situated in the follicular associated epithelium (FAE), the epithelial sheet overlaying Peyer's patches in the intestine [32].

Table 1
Differentiation into specific cell types can be induced by adding various compounds to the organoid medium.

Species	Medium	Compounds	Cell types	Ref.
Mouse	ENR	EGF, Noggin, Rspondin-1	CBC, Pc, TA, EC, GC, EEC, TC	[49]
	ENR-RankL	EGF, Noggin, Rspondin-1, rmRANKL	EC, M	[32]
	ENR-C	EGF, Noggin, Rspondin-1, CHIR	CBC, Pc, TA	[53]
	ENR-D	EGF, Noggin, Rspondin-1, DAPT	Pc, EC, GC, EEC	[49,53]
	ENR-I	EGF, Noggin, Rspondin-1, IWP2	EC, EEC	[53]
	ENR-V	EGF, Noggin, Rspondin-1, VPA	CBC, Pc	[53]
	ENR-CD	EGF, Noggin, Rspondin-1, CHIR, DAPT	Pc, EEC	[53]
	ENR-CV	EGF, Noggin, Rspondin-1, CHIR, VPA	CBC	[53]
	ENR-DV	EGF, Noggin, Rspondin-1, DAPT, VPA	CBC, Pc	[53]
	ENR-IV	EGF, Noggin, Rspondin-1, IWP2, VPA	EC	[53]
Human	EM	EGF, Noggin, Rspondin-1, A83, WNT3A, SB, Nicotinamide	CBC, Pc, TA	[49]
	DM	EGF, Noggin, Rspondin-1, A83	EC, GC, EEC, TC	[49]
	DM+RankL	EGF, Noggin, Rspondin-1, A83, IWP2, rhRANKL	EC, M	SM*
	DM+SCFA	EGF, Noggin, Rspondin-1, SCFA, (DBZ)	EC, L	[55,56]
	EM+CV	EGF, Noggin, Rspondin-1, A83, WNT3A, SB, CHIR, VPA	CBC, EEC	[53]
	EM+C	EGF, Noggin, Rspondin-1, A83, WNT3A, SB, CHIR	EC	[53]
	EM+CVNi	EGF, Noggin, Rspondin-1, A83, WNT3A, SB, CHIR, VPA, Nicotinamide	CBC, Pc	[53]

EGF, epidermal growth factor; CBC, crypt based columnar cell; Pc, Paneth cell; TA, transit amplifying cell; EC, enterocyte; GC, goblet cell; EEC, enteroendocrine cell; TC, tuft cell; M, M-cell; L, L-cell; RANKL, RANK-ligand; VPA, valproic acid; IWP2, inhibitor of WNT pathway 2; SCFA, short chain fatty acids; DBZ, Dibenzazepine; CHIR, CHIR99021; SB, SB202190. * unpublished data by SM.

Human organoids

Intestinal organoids of human origin can be established from a single biopsy. Crypts or single stem cells are isolated from the biopsy and cultured in matrigel, similar to mouse organoids [52,54]. For long-term culture, human organoids require expansion medium (EM), which contains ENR (EGF, Noggin, R-spondin), WNT3A, nicotinamide, n-acetylcysteine, B27, A83-01 (a TGFβ inhibitor) and SB202190 (an inhibitor of the kinase p38). Under these conditions, the organoids grow as cysts, mainly consisting of stem, Paneth and proliferating cells and can be expanded indefinitely. Upon withdrawal of WNT3A, nicotinamide and SB202190, differentiation into all mature intestinal epithelial cell types is induced, and a folded structure of the organoids becomes apparent [52]. The human organoid culture system is summarized in Fig. 2. Like murine organoids, human-derived organoid cultures can also be directed towards a specific fate by adjusting the culture medium (Table 1).

Organoids – an *in vitro* model of intestinal location and disease

In summary, organoids represent an unlimited source of intestinal epithelium, which is genetically stable and closely reflects the *in vivo* situation. Hence, organoids hold great potential as experimental tools, disease models, and as a stem cell source. To illustrate these various applications, we will discuss organoid cultures as a tool for studying the intrinsic epithelial properties from different intestinal locations, as a disease model for research of MVID and cystic fibrosis (CF), and finally as a possible source for stem cell transplantation.

Location-specificity of intestinal organoids

The epithelial organization of the small and large intestine changes along the cephalo–caudal axis in function, crypt-villus ratio, and cellular phenotypes. In the small intestine, various enzymes involved in food uptake are differentially expressed across duodenum, jejunum and ileum. The duodenum, for instance, is known to express proteins necessary for uptake of calcium and iron, which are absent in other regions of the small intestine [57]. The jejunum is mostly responsible for digestion and uptake of sugars, and the ileum for the transport of bile acid and uptake of vitamin B12 [57]. Various studies have described the expression patterns of these regional markers by either microarray or mRNA-sequencing, and thereby provide us with a comprehensive overview of the genetic make-up of each region in the gut [58,59].

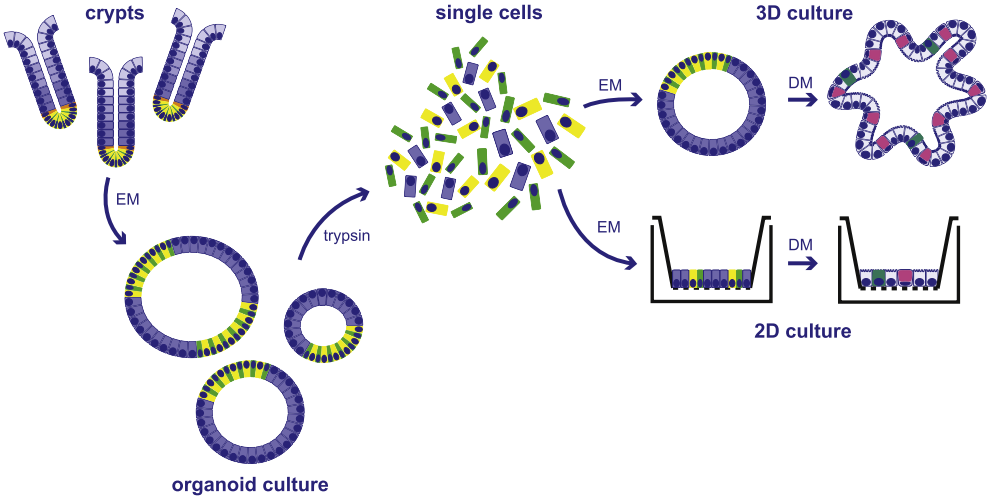

Fig. 2. Intestinal epithelial architecture and homeostasis in vivo and in vitro. Schematic representation of the generation and culture possibilities of human small intestinal organoids. Isolated crypts are cultured in expansion medium (EM) and consist mainly of CBC cells, Paneth cells and TA cells. After single cell processing by trypsin, cells can either be recultured as 3D organoids in matrigel or plated on a matrigel-coated transwell to generate a 2D monolayer of epithelial cells. Stems cells can be induced to differentiate by changing the medium. See text for details about expansion medium (EM) and differentiation medium (DM).

The fact that the intestine is so well adapted to its location-specific function, brings a new problem to the attention when organoids are considered as an experimental tool or disease model. An organoid model for a disease originally located in the ileum would have to maintain its ileum-specific gene expression profile *in vitro*.

In mice, the location-specific gene expression patterns have been shown to be regulated by the main transcription factor Gata4 [60,61]. Specifically, it was shown that Gata4 is expressed in all enterocytes and crypt cells of the duodenum and jejunum, while expression is lacking in distal ileum. Knockdown of Gata4 in an inducible mouse model, resulted in a diminished number of cells with a characteristic jejunal enterocyte expression pattern [60]. Moreover, the enterocytes in the jejunum predominantly adopt an expression pattern normally seen in enterocytes of the ileum [60].

To elucidate the source of regional identity in intestinal cells, we recently compared full mRNA libraries derived from duodenum, ileum and jejunum to material derived from *ex vivo* organoid cultures of these regions. This approach allowed us to conclude that (long-term) cultures of intestinal stem cells retain their location-specific gene expression patterns without the presence of their mesenchymal niche [59]. Therefore, the location-specific identity of small intestine epithelium is programmed in the intestinal stem cells, and maintained in organoid cultures. As such, the original location of the cultured material should be considered when using organoids as an experimental tool or disease model.

Recently, it has been demonstrated that colonic tissue of recipient mice can easily be prepared to engraft colonic [62], small intestinal [63] or fetus-derived small intestinal [64] organoids. These organoids have been shown to stably integrate into the recipient's colon where these appear to become fully functional tissue. Interestingly, the engrafted adult stem cells maintained their identity, whereas fetus-derived small intestinal organoids adopted a colonic phenotype upon engraftment. These data imply that in analogy with our report about the stable *in vitro* programming of adult stem cells, this programming is also maintained *in vivo*, at least in colonic tissue. Although we are unable to rule out the possibility of reprogramming of the intestinal stem cells by the mesenchymal niche of the small intestine upon engraftment, we foresee that if intestinal organoids are ever to be transplanted, it will likely be important to transplant cultures of all three parts of the small intestine in order to transplant all cephalocaudal functions of the intestine. Our opinion about organoids as a source for intestinal transplantation is discussed in more detail below.

Modelling disease in intestinal organoids

To date, diagnosis of many intestinal diseases is often based on either the pathological analysis of biopsies or genetic make-up of the patient. For most diseases this suffices for an accurate diagnosis, nonetheless the measure of intestinal function or dysfunction is more difficult in this manner. Since organoids retain patient-specific functional properties of epithelial cells, they could aid in diagnostics, unravelling pathogenesis and therapy modulation for patients suffering from intestinal diseases.

However, while addressing the human organoids as a model for intestinal diseases, one has to bear in mind that the organoids are only in part representing the intestine. The adult stem cells in the crypt are epithelial cells, indicating that the organoid model system falls short on interactions with other cell types such as fibroblasts, lymphocytes or glial cells. In complex intestinal diseases, such as inflammatory bowel disease (IBD), often multiple cell types are involved in the pathogenesis of the disease and therefore this disease might not be well represented in this model [65].

Nonetheless, organoids can still be helpful to elucidate the role of the epithelium in multifactorial diseases, since they enable the study of the epithelium without interference from non-epithelial cells. Moreover, by generating monolayers of cells from organoid cultures [66,67] (Fig. 2), it may be possible to generate co-cultures of various cell types, which enable the study of cellular interactions between e.g. lymphocytes and epithelial cells in the future.

In addition, organoids provide an excellent model for intestinal diseases that are based on epithelial defects, such as MVID. The histological phenotype found in biopsies from patients with MVID is retained in patient-derived organoid cultures [68]. Future studies on these patient-specific cultures could reveal the role of specific genes in cellular processes, such as intracellular trafficking or recycling of membrane proteins.

Next to the histological phenotype, also the functional phenotype of intestinal cells is preserved in organoid cultures, as demonstrated in organoids derived from patients with CF. Intestinal organoids

from CF patients show reduced organoid swelling in response to forskolin, which activates the CFTR channel through cAMP, compared to healthy controls [69]. By using this very sensitive functional assay, the degree of CFTR function/dysfunction is very well recapitulated in these organoids. In this way, organoids are patient-specific *in vitro* models of diseases, in contrast to *in vitro* colon cancer-derived cell models such as Caco2 cells or mouse models.

Testing patient-specific drug responses in organoids has already been shown for patients with CF and colon carcinoma [53,69,70]. Currently, the CFTR swelling assay is used in clinical trials to assess if *in vitro* drug response is in concordance with the patient's response *in vivo*.

Organoids – a safe stem cell source for transplantations?

Human small intestinal organoids have been shown to remain genetically stable in culture, and do not require genetic manipulation for immortalization [49]. Furthermore, the growth factors used for organoid culture mimic the *in vivo* stem cell niche, and as such represent a physiological environment. It thus seems that organoids represent a safe source for stem cell transplantations. However, for clinical application, adopted culture protocols that meet good manufacturing practice (GMP) standards have to be established. For example, matrigel is an undefined matrix derived from mice and WNT-conditioned medium is currently produced in cell lines that require fetal bovine serum, which are both not GMP grade. Currently, synthetic hydrogels, as alternative for matrigel [71], and synthetic proteins for WNT production are being tested to generate GMP grade organoid culture systems for future human applications.

Both autologous and allogeneic organoid transplantations could possibly be applied to patients suffering from an intestinal epithelial disease, such as MVID, IBD or short bowel syndrome (SBS). In case of allogeneic sources, stem cells could be isolated from an HLA-matched donor, possibly a close relative. Since a single intestinal donor biopsy is sufficient for stem cell isolation and amplification, this implicates a minimal invasive procedure for the donor. The recipient has to be treated with immunosuppressive therapy upon transplantation, in order to prevent graft rejection.

As an alternative approach, autologous organoid transplantations in combination with gene therapy could be applied. In that case, organoids should be grown from a biopsy of the patient itself, preventing the need for immunosuppressive therapy. Recently, gene therapy has successfully been applied to organoids grown from two CF patients [72]. In this study, the mutant allele of the *CFTR* gene was corrected *in vitro* using CRISPR/Cas9 mediated homologous recombination. Organoids with the corrected allele regained full functionality [72]. Conventional gene therapy approaches usually apply virus-mediated gene transfer. This harbours the risk of random integration events of the recombinant virus in the host genome, which in turn might activate oncogenes in the host genome. In the CRISPR/Cas9 gene therapy approach, plasmids were introduced into the host cells by transfection, which means they are only transiently expressed and only the homologous target sequence is stably integrated into the genome. Still, the guiding RNA (gRNA) used in this approach can potentially bind to sites with sequence homology to the target site, known as off-target sites, and induce insertions or deletions at these sites [73,74]. However, since organoid cultures can be established from a single stem cell, multiple clones could be analysed for off-targets after the gene correction, and only clones with the single desired genetic change could then be expanded into large amounts of organoids for transplantation purposes.

More recently, it was demonstrated that a novel method, called induced transduction by osmocytosis and propanebetaine (iTOP), can introduce Cas9 protein into cells without involvement of introducing DNA or mRNA into the target cells [75].

Further studies have to reveal if CRISPR/Cas9 or iTOP gene editing methods are safe for human applications. The potential procedure of autologous organoid transplantations in combination with gene therapy is illustrated in Fig. 3.

Organoid transplantations – a realistic therapy for MVID patients?

As discussed above, organoids can easily be established, grown in large amounts without immortalization, are genetically stable, and gene repair can be applied. As such, organoids represent a safe and

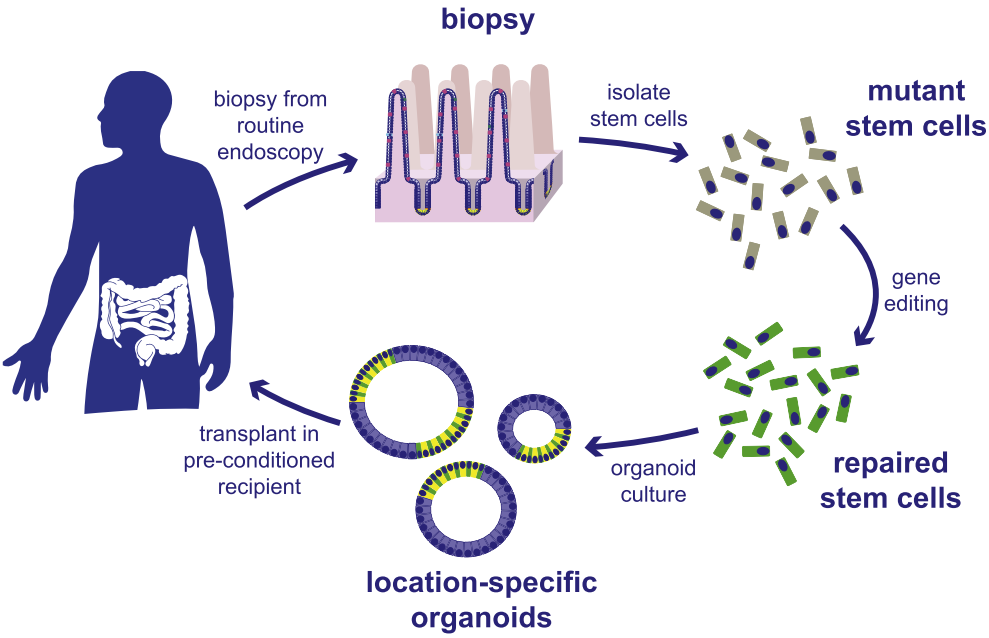

Fig. 3. Schematic illustration of autologous organoid transplantations in combination with gene therapy. Stem cells are isolated from a biopsy of a patient with an intestinal epithelial disease, the mutation is corrected *in vitro*, repaired stem cells are clonally grown up to organoids and subsequently transplanted back into the patient.

realistic source of adult stem cells for transplantation purposes. However, despite the success of organoid transplantation into colonic tissue of mice [62–64], our unpublished experiments have shown that organoid engraftment into the *in vivo* small intestine is a major hurdle.

Two processes seem to be crucial for successful organoid transplantations. First, the pre-existing (diseased) crypts have to be removed efficiently in the recipient. In the case of organoid transplantations in a mouse colon, this was achieved by DSS treatment and by mechanical damage of the epithelium using a cotton swab [62–64]. We have tried several methods for creating an epithelial niche in the small intestine, such as indomethacin treatment that could potentially be applied to patients. Additionally, a "scraper" attached to an endoscope could be applied for mechanical removal of the pre-existing epithelium.

The second crucial process is organoid engraftment into the lesions. Despite extensive efforts, we were not able to detect any engrafted donor organoids in the small intestines of transplanted mice. It is difficult to attribute this to a specific reason, since it is currently not possible to follow the fate of the organoids *in vivo* after the transplantations. In fact, it might be a combination of several small intestine-specific suboptimal conditions that together prevent organoid engraftment. This is substantiated by the notion that small intestinal organoids did engraft into the colon [63], and intestinal organoid units generated from pluripotent stem cells formed a mature and functional intestinal epithelium *in vivo* when transplanted under the kidney capsule [76] or into the peritoneal cavity [77]. Factors that negatively influence organoid engraftment into the small intestine might be peristalsis of the small intestine, a continuous secretion of mucus that might inhibit cell attachment, and the high intrinsic regenerative capacity of the endogenous intestinal epithelium that limits the time window of access to the open lesions.

It might therefore be necessary to step away from the intraluminal infusion of organoids, which we have been using so far (unpublished data SM). As an alternative method, organoids could be seeded onto a scaffold before transplantations. This would allow for an expansion of intestinal epithelial tissue *in vitro*, and the scaffold could also hold the organoids in place, and "pressed" against the denuded intestinal wall upon *in vivo* transplantations. Multiple biological and biocompatible synthetic scaffolds

are available for tissue engineering purposes [78]. Such a scaffold has successfully been used with mature intestinal epithelium, which was derived from dissociated rat foetal intestinal units. Such tissue-engineered neointestine (TENI) improved the symptoms of rats with a SBS when anastomosed to the remaining native intestine (reviewed in Ref. [79]).

In conclusion, the engraftment of organoids in the small intestine *in vivo* seems to be the major hurdle to overcome to apply organoids as a stem cell therapy for intestinal epithelial diseases. *In vitro* tissue engineering, i.e. seeding of organoids onto a tube-shaped scaffold, and subsequent transplantation of the scaffold into the denuded small intestine might be a new approach for further studies.

Future considerations for organoid transplantations

Previously, we have shown that mouse and human organoids derived from different locations along the small intestine (duodenum, jejunum and ileum) maintained their location-specific identity over a period of 10–12 weeks in culture, implicating that the location-specific epithelial functions are programmed in the stem cells [59]. This was substantiated by an *in vivo* study from Fukuda et al. where small intestinal organoids retained their small intestinal identity when heterotopically transplanted into the colon of recipient mice [63]. Together, these studies highlight a location-specific intrinsic program in the adult intestinal epithelial stem cells. This implicates that the different epithelial functions along the small intestine have to be restored separately in the case of organoid transplantations. Hence, donor material has to be established from duodenum, jejunum and ileum in order to restore complete intestinal function in the recipient. In case of local malabsorptive problems, such as SBS, ileum-derived organoids may suffice. Alternatively, organoids could beforehand be directed *in vitro* towards a certain fate and/or function prior to *in vivo* transplantations. Previously, we have shown that the fate of small intestinal stem cells can be manipulated *in vitro* (Table 1). Upon the addition of RANKL to the culture medium, organoids are induced to differentiate into functional M-cells [32]. Likewise, L-cells could be induced *in vitro* by the addition of short chain fatty acids to the culture medium of small intestinal organoids [55]. However, it remains elusive if such an *in vitro*-induced stem cell fate remains stable or converts back upon *in vivo* transplantations. Therefore, with the current knowledge, transplantations of stem cells derived from duodenum, jejunum and ileum of the donor would currently be the preferred method to restore all intestinal epithelial functions in a patient.

Summary

Organoids represent an exceptionally well-defined and controlled culture system to study patient-specific intestinal epithelial cells, which can also be used for elucidating disease mechanisms and functional testing of personalized medicine. Furthermore, stem cell based therapy is one of the future therapeutic approaches for paediatric enteropathies, which are currently incurable. Organoids are an interesting and presumably safe source of adult intestinal epithelial stem cells, but *in vivo* transplantation into the small intestine seems very challenging, and further studies are required to evaluate the potential of organoids for small intestinal regeneration.

Research agenda

- For clinical application, organoid culture protocols that meet GMP standards have to be established
- Clinical safety of gene editing by the CRISPR/Cas9 system has to be evaluated before autologous organoids can be considered for stem cell therapy
- Further studies are required to evaluate the potential of organoid transplantation for small intestinal regeneration

Practice points

- Intestinal organoids provide a location-*specific in vitro* model of the *in vivo* epithelium of the intestine
- Intestinal organoids provide a new tool to diagnose and study pathophysiology and treatment options for various (malabsorptive) intestinal diseases

Conflict of interest

No conflict of interest has been declared by the authors.

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