Modeling Development and Disease with Organoids

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http://dx.doi.org/10.1016/j.cell.2016.05.082

Recent advances in 3D culture technology allow embryonic and adult mammalian stem cells to exhibit their remarkable self-organizing properties, and the resulting organoids reflect key structural and functional properties of organs such as kidney, lung, gut, brain and retina. Organoid technology can therefore be used to model human organ development and various human pathologies 'in a dish.'' Additionally, patient-derived organoids hold promise to predict drug response in a personalized fashion. Organoids open up new avenues for regenerative medicine and, in combination with editing technology, for gene therapy. The many potential applications of this technology are only beginning to be explored.

In 1975, James Rheinwald and Howard Green described the first long-term culture of normal human cells (Rheinwald and Green, 1975). For this, they combined freshly isolated keratinocytes with irradiated mouse 3T3 fibroblasts, established in the same lab years earlier. As in stratified skin, cell division was confined to the basal layer of the growing clones, while superficial layers consisted of terminally differentiating keratinocytes that gradually developed a cornified cell envelope. Successive improvements in the methodology allowed the cultivation of large confluent sheets of epidermis grown from relatively small numbers of primary proliferative keratinocytes (for which the term ''stem cell'' was not applied). Green and co-workers performed the first successful treatment of two third-degree burn patients with cultured autologous keratinocyte sheets at the Peter Bent Brigham Hospital in 1980 (O'connor et al., 1981). In a particularly dramatic demonstration of the potential of the method, they showed, in the summer of 1983, that this approach was life-saving for the 5-year-old Jamie Selby and his 6-year-old brother Glen, who had both sustained burns over >95% of their body surface (Gallico et al., 1984).

In his own lab, Rheinwald built on this work to establish a comparable method for culturing another stratified squamous epithelium, the cornea (Lindberg et al., 1993). De Luca and Pellegrini applied this technology for the treatment of corneal blindness with a high rate of success, as reported upon long-term follow-up of 112 patients. Their procedure was straightforward: a 1–2 mm biopsy from the limbal region of the healthy eye was grown in culture on 3T3 feeder cells, and the resulting sheet was grafted onto the injured eye (Pellegrini et al., 1997; Rama et al., 2010). While the term ''organoid'' was not used in these pioneering studies, Rheinwald and Green were the first to reconstitute 3D tissue structure from cultured human stem cells.

Organoids revealed their first popularity in the years 1965–1985, shown by an increase in the PubMed search term ''organoids'' (Figure 1), mostly in classic developmental biology experiments that sought to describe organogenesis by cell dissociation and reaggregation experiments (for an overview, see Lancaster and Knoblich, 2014). The past 7–8 years have witnessed a revival of the organoid, yet in a somewhat different guise: an organoid is now defined as a 3D structure grown from stem cells and consisting of organ-specific cell types that self-organizes through cell sorting and spatially restricted lineage commitment (after Eiraku and Sasai, 2012; Lancaster and Knoblich, 2014).

Organoids can be initiated from the two main types of stem cells: (1) pluripotent embryonic stem (ES) cells and their synthetic induced pluripotent stem (iPS) cell counterparts and (2) organ-restricted adult stem cells (aSCs). Both approaches exploit the seemingly infinite expansion potential of normal stem cells in culture. For ES and iPS cells, here collectively termed pluripotent stem cells or PSCs, this potential has been an essential prerequisite for their discovery. By contrast, aSCs—with the exception of Green's skin cells—were long believed to be incapable of significant proliferation outside of the body. Yet, recent years have witnessed the rapid development of growth factor cocktails that mimic the various organ stem cell niches. When PSCs and aSCs are allowed to differentiate in culture, they display an uncanny capacity to self-organize into structures that reflect crucial aspects of the tissues to which they are fated.

Organoids Derived from Pluripotent Stem Cells

Ever since pluripotent ES and iPS cell lines were established, scientists have applied insights from developmental biology to derive differentiated cell types from these stem cells (Chen et al., 2014; Cherry and Daley, 2012) (Figure 2). Yoshiki Sasai and his colleagues were the first to take this one step further by asking whether such an in vitro system could recapitulate some of the robust regulatory systems of organogenesis—in terms of not only cell differentiation, but also spatial patterning and morphogenesis. In a remarkable tour de force, they developed methods to generate brain structures, retina, and pituitary 'in a dish' (Eiraku and Sasai, 2012).

Brain Organoids

The central nervous system derives from the neural ectoderm. Set up first as the neural plate, it is then shaped into the neural tube through folding and fusion. Morphogen gradients in this

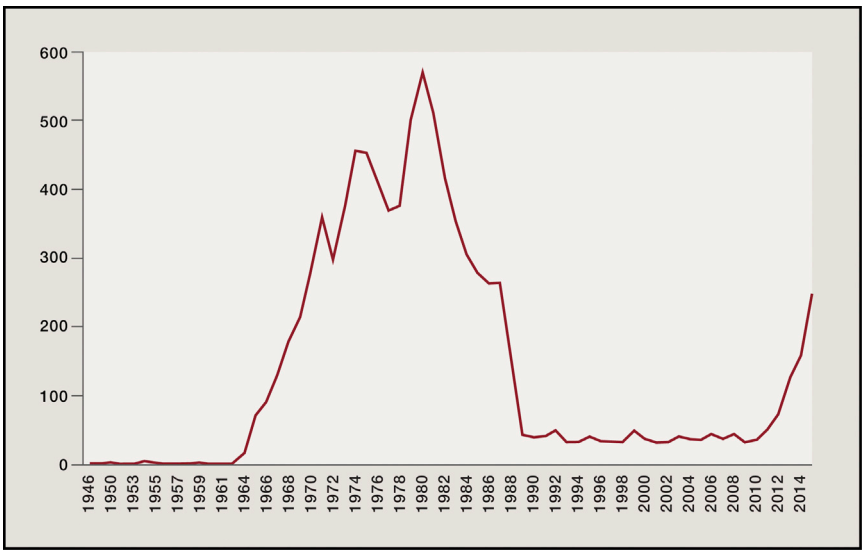

Figure 1. Citations to the Search Term "Organoids" in PubMed

tube establish a dorsal-ventral axis (roof, alar, basal, and floor plate) and a rostral-caudal axis (tel-, di-, mes-, and rhombencephalon and spinal cord). Neurons are generally generated from neural stem cells that reside near the ventricles. These stem cells initially increase their numbers through symmetric divisions. During neurogenesis, stem cells switch to asymmetric divisions to yield temporal waves of distinct self-renewing progenitors and differentiated cell types, such as neurons and intermediate progenitors, that migrate outward to generate region-specific stratified structures such as the medulla, the optic tectum, and the cerebral cortex (see Eiraku and Sasai, 2012; Lancaster and Knoblich, 2014).

Spontaneous neural differentiation occurs in ES culture in the absence of inhibitors of neural differentiation (such as BMP, Nodal, and Wnts), consistent with a neural-default state for ES cells. Based on this notion, Sasai and colleagues designed SFEBq: serum-free floating culture of embryoid body-like aggregates with quick reaggregation (Eiraku et al., 2008). In this culture system, ES cells isolated from growth factor-free 2D cultures are reaggregated in 96-well non-adhesive culture plates. The re-aggregates are kept in serum-free medium containing no or minimal growth factors for 7 days, after which they are replated in adhesion plates. When lumens form, the ES cells differentiate and polarize to form a continuous neurectoderm-like epithelium that subsequently generates stratified cortical tissues containing cortical progenitors, deep cortical-layer neurons, superficial cortical-layer neurons, and so called Cajal-Retzius cells. In the absence of growth factors, the generated cortical tissue spontaneously adopts a rostral hypothalamic fate. Regional identity (e.g., olfactory bulb, rostral and caudal cortices, hem, and choroid plexus) can be selectively controlled by addition of specific patterning factors such as Fgf, Wnt, and BMP. It was thus shown that, under controlled conditions, ES cells can recapitulate some of the spatial and temporal events leading to the formation of layered neural structures in the brain.

Lancaster and Knoblich took this approach to a next level by generating cerebral organoids, or "mini-brains": single neural organoids containing representations of several different brain regions (Lancaster et al., 2013). Like the Sasai method, the approach starts with floating embryoid bodies, but growth factors are not added to drive particular brain region identities. Instead, the aggregates are embedded in a laminin-rich extracellular matrix secreted by the Engelbreth-Holm-Swarm tumor cell line (Matrigel). This allows outgrowth of large neuroepithelial buds, which spontaneously develop into various brain regions. Cerebral organoids can reach sizes of up to a few millimeters when grown in a spinning bioreactor. A spectacular variety of brain regions, including retina, dorsal cortex, ventral forebrain, midbrain-hindbrain boundary, choroid plexus, and hippocampus, is observed in these cultures (Figure 4A).

A subsequent study applied single-cell RNA sequencing to compare gene expression programs of cells within cerebral organoids to those of fetal human neocortex development (Camp et al., 2015). It was thus found that gene expression programs of cortical cells in organoids are remarkably similar to those of the corresponding fetal tissue, underscoring that aspects of human cortical development can be studied in organoid culture.

Retinal Organoids

The retina is of neurectodermal origin and constitutes the light-receptive neural region of the eye. The optic vesicle forms as a pseudostratified, cystic outgrowth of the diencephalon. The front of the vesicle then moves inward to form the two-layered optic cup, consisting of the outer retinal pigment epithelium and the inner neural retina. The neural retina continues to stratify into layers of photoreceptors and supportive cell types, such as horizontal cells, bipolar cells, and amacrine cells.

To mimic this process in vitro (Eiraku et al., 2011), Sasai and colleagues again generated floating embryoid bodies from re-aggregated murine ES cells in growth factor-free medium to generate neuroectoderm. Matrigel, dissolved in the medium, allowed the formation of more rigid neuro-epithelial tissues. This resulted in the formation of buds of retinal primordial tissue resembling the optic vesicle. Isolated buds were then maintained in a medium supporting retinal tissue identity. The morphological tissue shape changes were reminiscent of the stepwise evagination and invagination of the optic cup in vivo. Retinal stratification with proper apical-basal polarity occurred, and markers of neural retina and pigment epithelium were expressed in a spatially correct manner. More recently, optic cup organoids were generated from human PSCs (Nakano et al., 2012). These human retinal organoids resembled their mouse counterparts but encouragingly also displayed human-specific features: they are larger in size (yet still small relative to the "real thing"), and the formed neural

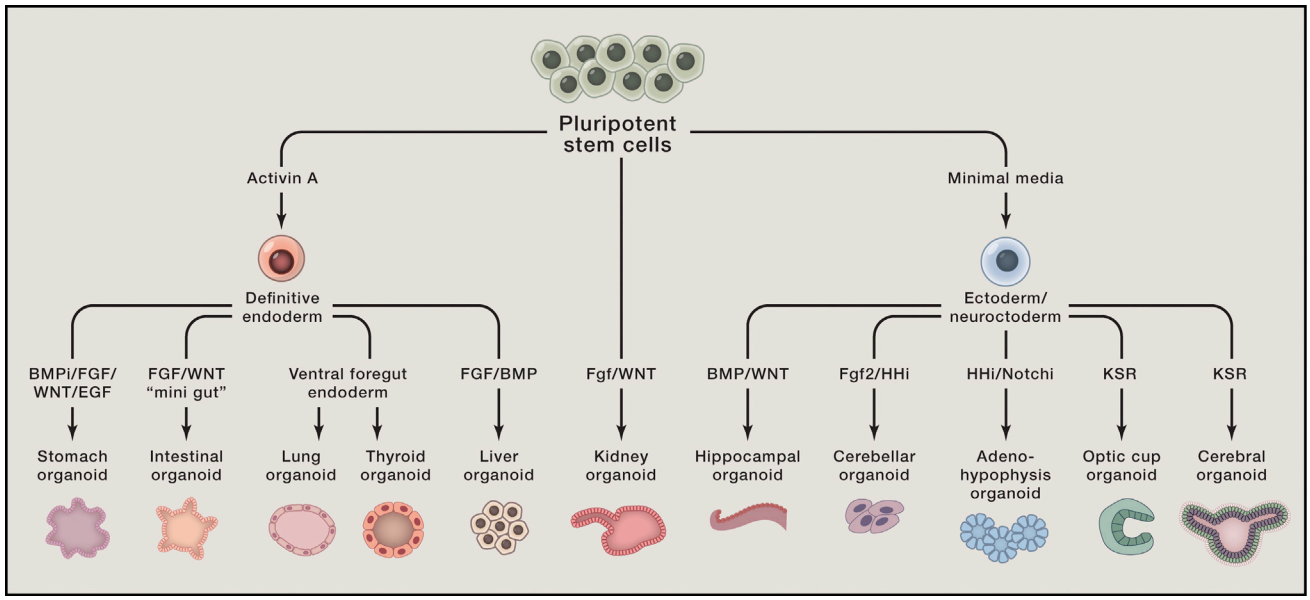

Figure 2. Schematic of the Various Organoids that Can Be Grown from PSCs and the Developmental Signals that Are Employed
Adapted from Lancaster and Knoblich, 2014.

retina grows into a thick multi-layered tissue containing both rods and cones, whereas cones were rarely observed in mouse organoid cultures.

Adenohypophysis Organoids

The adenohypophysis secretes multiple systemic hormones. During early mammalian development, its anlage originates as a placode in the non-neural head ectoderm near the anterior neural plate. The thickened placode invaginates and detaches from the oral ectoderm, forming a hollowed epithelial vesicle, Rathke's pouch. This process depends on poorly defined cross-signaling between ectoderm and developing neural tube. Sasai's group sought to recapitulate the inductive microenvironment of this morphogenetic field in order to promote the simultaneous generation of both tissues within the same aggregate of SFEBq-cultured ES cells. Three-fold larger cell aggregates were required, compared to the above protocols. Hedhehog and Notch antagonists were added to block neural fate in the outer layers and to allow the subsequent development of all major hormone-producing linages, respectively. Under these conditions, ES cells differentiated into head ectoderm and hypothalamic neuroectoderm in adjacent layers within the aggregate. Rathke's-pouch-like structures arose at the interface of these two epithelia, and the various endocrine cell types were subsequently formed. Upon transplantation under the kidney capsule of hypophysectomized mice, the aggregates partially rescued systemic glucocorticoid level and prolonged survival of the mice.

Cerebellar Organoids

The initial phase of cerebellar development depends on the function of the isthmic organizer located at the midbrain-hindbrain boundary. Sasai and colleagues focused on the induction of isthmic development in an attempt to create functional Purkinje cells, the beautiful key output cells of the cerebellum. Again, they started from a mouse SFEBq culture. In order to produce caudal brain structures, Fgf2 was added soon after initiation of the culture. To dorsalize the caudalized brain organoids, a Hedgehog inhibitor was added during the second week. These conditions recapitulated early cerebellar plate development, eventually leading to the formation of mature Purkinje cells (Muguruma et al., 2010). In a subsequent study, the investigators reported that the addition of Fgf19 and SDF1 to this protocol allows human ES cells to generate a polarized structure reminiscent of the first trimester cerebellum (Muguruma et al., 2015).

Hippocampus

The hippocampus develops from the dorsomedial telencephalon through a precursor structure termed the medial pallium. A final protocol developed by Sasai and coworkers involved the in vitro generation of a reliable source of hippocampal tissue from human ES cells (Sakaguchi et al., 2015). SFEBq served once again as the starting material. Stimulation by BMP and Wnt induced choroid plexus, the dorsomedial-most part of the telencephalon. Careful titration of BMP and Wnt exposure allowed the self-organization of tissue resembling the medial pallium, located adjacent to choroid plexus in the developing brain. Following long-term dissociation culture, granule neurons and pyramidal neurons were formed, both of which were electrically functional within connected networks.

In addition to these CNS organoids, protocols have also been developed to grow various endodermal organoids from PSCs. Formation of the endoderm germ layer during gastrulation requires Nodal signaling. Definitive endoderm presents as a 2D sheet of cells, which is subsequently patterned along the anterior-posterior axis and folded into a primitive gut tube, from which all endodermal organs arise. The foregut forms the anterior section of this tube and generates, e.g., the thyroid, lungs, stomach, liver, and pancreas. The mid- and hindgut develop into small intestine, colon, and rectum. Insights into the signals

that control these fate decisions in vivo can be exploited in vitro. Exposure to Nodal or its mimetic Activin directs differentiation of PSCs into definitive endoderm and serves as a common starting point of these protocols. Exposure to subsequent inductive signals can then induce the various endodermal organ identities (reviewed in Sinagoga and Wells, 2015).

Stomach Organoids

The stomach develops from the posterior foregut. Wells and colleagues used activin treatment of human PSCs to generate definitive endoderm (McCracken et al., 2014). Subsequent addition of BMP inhibitors and of FGF and Wnt activators instructed the cells toward a foregut fate. When retinoic acid was applied, the organoids were specified toward a posterior foregut fate. Finally, high concentrations of EGF then converted these into human gastric organoids, progressing through molecular and morphogenetic stages that resembled those of the developing antrum of the mouse stomach. Organoids contained primitive gastric gland- and pit-like domains, proliferative zones with $Lgr5^+$ stem cells, mucous cells, and a host of gastric endocrine cells.

Lung and Thyroid Organoids

The lung and the thyroid arise from $Nkx2\text{-}1^+$ progenitors in the developing ventral foregut endoderm. An initial study demonstrated the directed differentiation of primordial lung and thyroid progenitors from ESCs. The protocol involves activin-induced definitive endoderm and treatment with TGFβ /BMP inhibitors, followed by BMP/FGF stimulation, and results in a relatively pure population of progenitors that recapitulate early developmental milestones of lung/thyroid development (Longmire et al., 2012). This study has been the stepping stone for subsequent attempts to create organoids representing mature versions of the two organs.

A first description of the generation of lung organoids from iPS cells was reported by Rossant and colleagues and involved at its last stage air-liquid interphase culture. The protocol was applied to CFTR mutant iPS cells as a proof of concept for modeling cystic fibrosis (Wong et al., 2012). Snoeck and colleagues designed an improved four-stage, 50-day protocol (Huang et al., 2014). First, definitive endoderm was induced by Activin A. Subsequently, anterior foregut endoderm was induced by sequential inhibition of BMP, TGF-β, and Wnt signaling. The cells were then ventralized by Wnt, BMP, FGF, and RA to obtain lung and airway progenitors. Finally, epithelial cell types (basal, goblet, Clara, ciliated, type I and type II alveolar epithelial cells) were matured using Wnt, FGF, c-AMP, and glucocorticoids. Spence and colleagues (Dye et al., 2015) similarly started from Activin-treated human PSCs but then followed a slightly different trajectory. Subsequent addition of TGFβ/BMP inhibitors, FGF4, and Wnt activators instructed the cells toward an anterior foregut fate. When the Hedgehog pathway was simultaneously activated, organoids were ventrally specified toward a lung fate. Upon embedding in Matrigel and prolonged exposure to Fgf10, mature lung organoids arose. The cultures could be maintained for several months and resembled proximal airways, containing basal cells, ciliated cells, and Clara cells. The endodermal airway tissues were found to be often surrounded by smooth muscle actin (SMA)-positive mesenchymal cells. Early markers of the distal (alveolar) airways were expressed early in culture but were lost later.

Initial attempts to create thyroid organoids involved forced expression of the lineage-specific transcription factors NKX2.1 and PAX8 and encouragingly resulted in the formation of mouse and human thyroid follicles in vitro and upon transplantation (Antonica et al., 2012; Ma et al., 2015). Kotton and colleagues applied an improved version of their "all soluble factor" protocol (Longmire et al., 2012), followed by sequential treatment by BMP4/FGF2 and induced maturation by 3D plating in the presence of thyroid-stimulating hormone in Matrigel. The resulting fully mature murine thyroid follicular organoids secreted thyroid hormones in vivo upon transplantation and rescued hypothyroid mice. The same protocol allowed the derivation of human thyroid progenitors from iPS cells (Kurmann et al., 2015).

Small Intestinal Organoids

Wnt and FGF signals are known to specify definitive endoderm toward mid-/hindgut fates ("posteriorization"). To generate intestinal organoids (McCracken et al., 2014; Spence et al., 2011), Activin-treated human PSCs were cultured with FGF4 and WNT3a. Mid/hindgut spheroids budded off from the 2D monolayer epithelium and were further cultured in Matrigel along with a pro-intestinal growth factor cocktail, defined previously for expansion of adult crypt cultures (Sato et al., 2009; see below). The organoids expanded over 1–3 months to give rise to a polarized intestinal epithelium patterned into villus-like structures and crypt-like proliferative zones and containing all major epithelial cell types. Intestinal mesenchyme (presumably derived from mesodermal remnants after endoderm induction) surrounded the epithelial structures and consisted of myofibroblasts and smooth muscle cells (McCracken et al., 2014; Spence et al., 2011). Transplantation of these organoids into immunodeficient mice yielded human epithelium and laminated human mesenchyme, supported by mouse vasculature. The transplanted tissue was functional, as shown by permeability and peptide uptake tests (Watson et al., 2014).

Liver Organoids

During early hepatogenesis, progenitor cells delaminate from the foregut endoderm to form a condensed tissue mass termed the liver bud, which is vascularized soon thereafter. Taniguchi and co-workers exploited cross-signaling between endodermal epithelial, mesenchymal, and endothelial progenitors in an effort to generate tissues reminiscent of the human liver bud. Human PSCs were induced into hepatic endodermal cells in 2D culture (a protocol involving activin treatment, followed by bFGF/BMP4). The human PSC-derived hepatic cells were mixed with mesenchymal stem cells and endothelial cells. Plated at high density on a layer of Matrigel, 3D aggregates spontaneously formed (Takebe et al., 2013). These liver bud-like aggregates contained blood vessels that, upon transplantation into mice, connected to the host vessels within 48 hr. Liver-specific functions such as protein production and human-specific drug metabolism became evident over time. Furthermore, mesenteric liver bud transplantation rescued recipient mice from drug-induced lethal liver failure.

The Mesodermal Kidney

The kidney, with its more than 20 specialized cell types, exhibits the highest architectural complexity of all organs outside of the CNS. The adult kidney, or metanephric kidney, arises from the

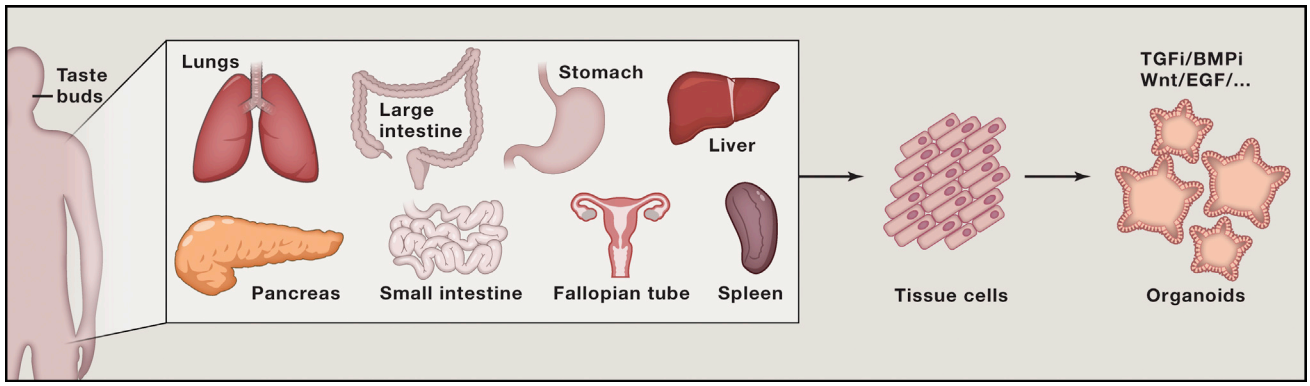

Figure 3. Schematic of the Various Regions of the Body that Can Be Cultured as aSC-Derived Organoids

posterior end of the embryonic intermediate mesoderm, which in turn derives from the primitive streak (presomitic mesoderm). The intermediate mesoderm generates the two key kidney progenitor populations: the ureteric epithelium and the metanephric mesenchyme. Through reciprocal interactions, these form the collecting ducts and nephrons (i.e., the epithelia of glomeruli and proximal and distal renal tubules), respectively. Until recently, the complex spatial and temporal control of organogenesis has stood in the way of a detailed molecular understanding of specification of individual cell types. Despite this, rapid progress has been made in establishing protocols for differentiation of human PSCs into virtually complete "mini-kidneys."

First, in 2013, it was shown how to induce intermediate mesoderm from PSCs under defined media conditions (Mae et al., 2013). One of the renal precursor tissues that derives from the intermediate mesoderm, the ureteric epithelium, can be generated from human PSCs in 2D via a similar mesodermal specification step (Xia et al., 2013). Upon aggregation with dissociated mouse embryonic kidney, these progenitors self-organize into 3D ureteric bud structures. The second renal precursor tissue, the metanephric mesenchyme, can be created from human and mouse embryoid bodies through sequential exposure to defined soluble factors. Coculturing of the resulting metanephric mesenchyme with spinal cord tissue, a nephric inducer, produces well-organized nephric tubules and nascent glomeruli (Taguchi et al., 2014).

Little and colleagues managed to balance the two divergent commitment paths to produce both principal lineages of the kidney simultaneously (Takasato et al., 2014). Their original protocol involves the application of Activin A and Bmp4 to human PSCs cultured in 2D to generate primitive streak identity. Fgf9 drives these cells toward an intermediate mesoderm identity, after which they spontaneously develop further into ureteric bud and metanephric mesenchyme. The cells display 3D morphologies when grown at low density in 2D or when cocultured with mouse kidney reaggregates. In both cases, structures resembling ureteric epithelium and proximal tubules appear. In a spectacular follow-up study, the protocol was further refined and simplified: human PSCs are cultured in 2D in the presence of Wnt signals for 4 days followed by 3-day exposure to Fgf9. After this, the cells are pelleted and cultured as 3D organoids for up to an additional 3 weeks. Numbers of nephrons are strongly increased upon a brief (1 hr) exposure to a Wnt agonist at the start of organoid culture. A complex multicellular kidney organoid results that contains fully segmented nephrons and is surrounded by endothelia and renal interstitium (Figure 4B). Kidney organoids may contain >500 nephrons with defined glomeruli comprising a Bowman's capsule with podocytes and connected to proximal tubules. Occasionally, glomeruli show evidence of endothelial invasion.

While remarkably complete, further improvements of the protocol will focus on tubular functional maturation, more extensive glomerular vascularization, and the formation of a contiguous collecting ductal tree "with a single exit path for urine" (Takasato et al., 2015).

Organoids Derived from Adult Stem Cells

While PSC-based organoids exploit developmental processes for their establishment, aSCs can be coerced to form organoids by creating conditions that mimic the stem cell niche environment during physiological tissue self-renewal or during damage repair (Figure 3). As first described for gut stem cells (Korinek et al., 1998), the Wnt pathway has emerged as the major driver of epithelial aSCs (Clevers et al., 2014). Lgr5 (a receptor for the secreted Wnt-amplifying R-spondins and itself encoded by a Wnt target gene) marks active aSCs in many, if not all, epithelia. It is not surprising that Wnt activators (Wnt3A, R-spondins, or the small molecule GSK3 inhibitor CHIR) are key components of most aSC culture protocols and that Lgr5$^+$ stem cells invariably appear in such cultures. Below, I discuss the establishment of feeder layer/serum-free, fully defined 3D culture conditions for a rapidly growing list of epithelial tissues.

Small Intestine and Colon

The small intestinal epithelium displays an extremely short turnover time of ~5 days. Actively proliferating Lgr5$^+$ intestinal stem cells reside at the crypt base (Barker et al., 2007). Their rapidly dividing, transit-amplifying (TA) daughter cells occupy the remainder of the crypts and, upon differentiation, move onto the flanks of the villi to eventually die at the villus tips. Differentiated cell types include absorptive enterocytes, multiple secretory cell types (Paneth cells, goblet cells, enteroendocrine

cells, and tuft cells), and the M cells that cover Peyer's patches (Clevers, 2013).

Crypt stem cells are tightly controlled by four signaling pathways. Wnt constitutes the key pathway to maintain stem cell fate and drive proliferation of stem and TA cells. Notch helps to maintain the undifferentiated state of proliferative stem and TA cells: when Notch signaling is blocked, the cells instantly differentiate into goblet cells. Epidermal growth factor (EGF) signals exert strong mitogenic effects on stem and TA cells. And finally, BMP signals are active in the villus compartment, and their inhibition is crucial to create a crypt-permissive environment (Clevers, 2013).

Encouraged by the observation that Lgr5 crypt stem cells can go through thousands of cell divisions in vivo, we established a culture system that allows growth of epithelial organoids ("mini-guts") from a single Lgr5 stem cell (Sato et al., 2009). Whole crypts or single Lgr5 stem cells are suspended in Matrigel and are cultured in serum-free medium supplemented with three recombinant proteins: R-spondin-1 (a Wnt signal amplifier and ligand of Lgr5), EGF, and the BMP inhibitor Noggin. For colon crypt culture, Wnt3a is additionally required because colon epithelium itself makes little, if any, Wnt. The organoids strictly consist of a simple highly polarized epithelium, tightly closing off a central lumen. Crypt-like structures project outward. The basal side of the cells is oriented toward the surrounding Matrigel. Enterocyte brush borders form the luminal surface, while secretion by Paneth and goblet cells occurs toward the lumen. All cell types of the epithelium are represented at normal ratios (Grün et al., 2015; Sato et al., 2009). The organoids can be passaged weekly at a 1:5 ratio for years and are remarkably stable, both genetically and phenotypically (Sato and Clevers, 2013).

Addition of Wnt3A to the combination of growth factors allowed seemingly indefinite growth of mouse colon organoids. Addition of nicotinamide, along with a small molecule inhibitor of Alk and an inhibitor of p38, was required for long-term culture of human small intestine and colon organoids (Jung et al., 2011; Sato et al., 2011). Given that Lgr5 protein expression is vanishingly low, other stem cell markers have been explored to initiate intestinal organoid cultures, including CD24 (von Furstenberg et al., 2011), EphB2 (Jung et al., 2011), and CD166+/GRP78− (Wang et al., 2013). As proof of stability upon culture, a large batch of organoids was grown from a single Lgr5 colon stem cell and transplanted per anum into multiple mice with experimental colitis. The organoids readily integrated as functional epithelial patches that were indiscernible from the surrounding host epithelium (Yui et al., 2012).

In a different approach, fragments of neonatal mouse intestine containing epithelial and mesenchymal elements were grown in serum-containing medium (without specific growth factors) in collagen with air-liquid interface. The expanding cystic structures consisted of a simple epithelium in which all cell types were discernible. The structures were surrounded by myofibroblasts and were responsive to R-spondin and to Notch inhibition (see above) (Ootani et al., 2009).

The mini-gut culture system has since been adapted for the generation of organoids representing the epithelial compartments of a series of mouse and human tissues of ecto-, meso- and endodermal origin. The essential components appear to be: (1) a potent source of Wnt, (2) a potent activator of tyrosine kinase receptor signaling like EGF, (3) inhibition of BMP/Tgfβ signals, and (4) Matrigel. It is not essential to start from purified Lgr5$^+$ aSCs. Small fragments of primary tissue serve well as starting material, possibly due to the fact that the culture conditions mimic a damage response, which in many tissues can recruit committed cells back to a stem cell state (Clevers, 2015)

Stomach

Rapidly proliferating Lgr5 stem cells are located at the base of pyloric glands of the adult mouse stomach. With slight modifications to the mini-gut culture system, single Lgr5 cells efficiently generated long-term, continuously expanding organoids closely resembling mature pyloric epithelium (Barker et al., 2010). At the base of glands of the gastric corpus, Troy marks specialized Chief cells. In a remarkable example of cellular plasticity, these Chief cells spontaneously dedifferentiate to act as multipotent epithelial stem cells in vivo, particularly upon damage. Single Troy$^+$ chief cells can be cultured to generate long-lived gastric organoids, containing the various cell types of corpus glands (Stange et al., 2013). Very similar conditions have allowed long-term culturing of human stomach organoids that maintain many characteristics of the original tissue (Bartfeld et al., 2015).

Liver and Pancreas

The two cell types of the liver proper (the hepatocyte and the bile duct cell) turn over at a slow, pedestrian rate. In agreement, Lgr5 is not expressed at appreciable levels in the healthy adult mouse liver. Yet, we observed that small Lgr5$^+$ cells appear near bile ducts upon toxic damage, coinciding with robust Wnt pathway activation. These damage-induced Lgr5$^+$ cells generated hepatocytes and bile ducts in vivo. When cultured in a modified version of the mini-gut medium, single Lgr5$^+$ cells could be clonally expanded as organoids, consisting largely of progenitor cells expressing early bile duct and hepatocyte markers. Removal of mitotic stimuli and simultaneous inhibition of Notch signals led to hepatocyte lineage differentiation. Upon transplantation, these organoids matured into functional hepatocytes (Huch et al., 2013b). In a follow-up study, we defined conditions for long-term expansion of adult bipotent progenitor cells from human liver. Somewhat surprisingly, one-third of all mature bile duct cells could initiate clonal liver organoid growth. Deep sequencing of clonal organoids derived at different intervals of culture revealed a highly stable genome at the structural level, while single base changes occurred at very low rates. Again, the cells could be converted into functional hepatocytes in vitro and upon transplantation into mice (Huch et al., 2015). The same protocols allowed long-term expansion of canine liver progenitor cells that could be differentiated toward functional hepatocytes (Nantasanti et al., 2015).

Like their liver counterparts, the exocrine/acinar, ductal, and endocrine cell types of the adult pancreas turn over very slowly. Wnt signaling is inactive and Lgr5 is not expressed under physiological conditions, yet the Wnt pathway is robustly activated upon injury, concomitant with induced Lgr5 expression in regenerating pancreatic ducts. Under modified mini-gut conditions, single isolated duct cells could be cultured long-term as pancreatic progenitor organoids. Clonal pancreas organoids differentiated along ductal and endocrine lineages when grafted

in vivo in a developing pancreas, indicative of bipotentiality (Huch et al., 2013a). Similar observations were made for human pancreatic organoids (Boj et al., 2015). Grompe and colleagues addressed the identity of the organoid-initiating epithelial cell from mouse pancreas and liver using a set of cell surface markers and found that the transcriptomes of the two populations overlapped extensively. Pancreatic organoid cells had the unexpected capacity to generate hepatocyte-like cells upon transplantation in a mouse liver damage model, indicative of the close kinship of these two progenitor populations (Dorrell et al., 2014).

Prostate

The pseudostratified prostate epithelium consists of basal and luminal cells. We developed a mini-gut-based 3D culture protocol that supports long-term expansion of primary mouse and human prostate organoids, composed of fully differentiated basal and luminal cells. Single human luminal as well as basal cells gave rise to organoids, yet luminal-cell-derived organoids more closely resembled prostate glands. Stimulation with R-spondin/Wnt was not essential for continued growth of the organoids but strongly induced luminal cells, leading to a prostate-like pseudostratified structure of the organoids. Long-term cultured organoids were genetically stable and reconstitute prostate glands in recombination assays (Karthaus et al., 2014). Independently, Shen and colleagues developed a Matrigel/EGF-based culture system supplemented with androgens and reported very similar observations (Chua et al., 2014).

Mammary Gland

This pseudostratified epithelium consists of two major cell lineages. The inner (luminal) cells secrete milk, while the contractile outer layer of myoepithelial (basal) cells ejects the milk. No long-term organoid protocol has been reported yet. However, encouragingly, freshly isolated human mammary epithelial cells have been cultured for two to three passages in floating collagen gels in the presence of a Rho-associated kinase inhibitor to form branching ducts with alveoli at their tips. Basal and luminal markers were expressed at correct positions, and the ducts displayed contractility. Thus, the organoids resembled terminal ductal-lobular units, the functional units of the mammary gland (Linnemann et al., 2015). Since Wnt signals and Lgr5 have been implied in mammary stem cell biology (Plaks et al., 2013; Rios et al., 2014), it will be of interest to test the effects of the addition of Wnt/R-spondin to these cultures.

Fallopian Tube

The fallopian tube of the uterus is lined by a simple columnar epithelium in which secretory cells produce tubular fluid, while ciliated cells facilitate transport of gametes. Since the epithelium is exposed to cyclical hormonal changes, self-renewal mechanisms are of critical importance for its integrity. Notably, recent evidence has indicated that the fallopian tube epithelium is the tissue of origin for ovarian cancer. Based on the mini-gut-protocol, long-term, stable 3D organoid cultures were established from human fallopian tubes. Single epithelial stem cells gave rise to clonal organoids containing both ciliated and secretory cells, thus establishing an experimental system for the study of the human fallopian tube epithelium in health and disease (Kessler et al., 2015).

Taste Buds

Previous studies had shown that Lgr5 marks adult stem cells in the rapidly self-renewing taste buds of the tongue. Using the original mini-gut culture protocol, single isolated stem cells from taste tissue generated continuously expanding 3D organoids, which phenotypically contained mature taste receptor cells (Ren et al., 2014). To assay functionality of these cells, cultured organoids were reseeded in 2D onto laminin-coated coverslips in the same culture medium. By calcium imaging assays, dose-dependent responses to tastants were readily documented, demonstrating that functional taste cells can be generated ex vivo from single Lgr5+ taste bud stem cells. Moreover, it could be concluded that single stem cells generate all taste cell types and that the formation of taste cells does not require innervation.

Lung

Hogan and colleagues reported an early bronchiolar lung organoid culture protocol, involving Matrigel supplemented with EGF, e.g. Single basal cells isolated from the trachea grew into ''tracheospheres'' consisting of a pseudostratified epithelium with basal cells and ciliated luminal cells. These organoids could be passaged at least twice. No mature Clara-, neuroendocrine- or mucus-producing cells were observed (Rock et al., 2009). In a later study, this clonal 3D organoid assay was used to screen for factors controlling generation of ciliated versus secretory cells from basal cells. It was thus found that IL-6 treatment resulted in the formation of multiciliated cells at the expense of secretory and basal cells (Tadokoro et al., 2014). Figure 4C depicts a human airway organoid.

Organoids representing the distal airways (''alveolospheres'') have been more recently established. The alveoli consist of gas-exchanging type I and surfactant-secreting type II cells. While both cell types originally derive from a common progenitor, it appears that, later in life, a rare self-renewing type II cell acts as the stem cell to regenerate the alveolar epithelium. Indeed, sorted type II cells remained proliferative in short-term culture and could generate type I cells (Desai et al., 2014; Treutlein et al., 2014). Alternative culture conditions allowed establishment of mouse and human alveolospheres from single type I as well as type 2 alveolar cells, containing both cell types in the same organoid. Having said this, these alveolosphere culture conditions are as yet not fully defined, requiring co-culture with non-epithelial cells (e.g., mouse lung fibroblasts) (Barkauskas et al., 2013; Jain et al., 2015).

Salivary Gland

Coppes and colleagues have exploited organoid culture to expand single salivary gland cells in vitro into distinct lobular or ductal/lobular organoids, containing some salivary gland lineages. The original short-term culture technology depended on FGF, EGF, and Matrigel. The cultured cells were able to efficiently restore radiation-damaged salivary gland function in transplanted mice (Nanduri et al., 2014). In a follow-up study, robust Wnt pathway activation through the addition of Wnt3A and R-spondin allowed long-term expansion of the organoids, containing all differentiated salivary gland cell types. Transplantation of these cells into submandibular glands of irradiated mice robustly restored saliva secretion and increased the number of functional acini in vivo (Maimets et al., 2016). Since post-radiation hyposalivation often leads to irreversible and untreatable

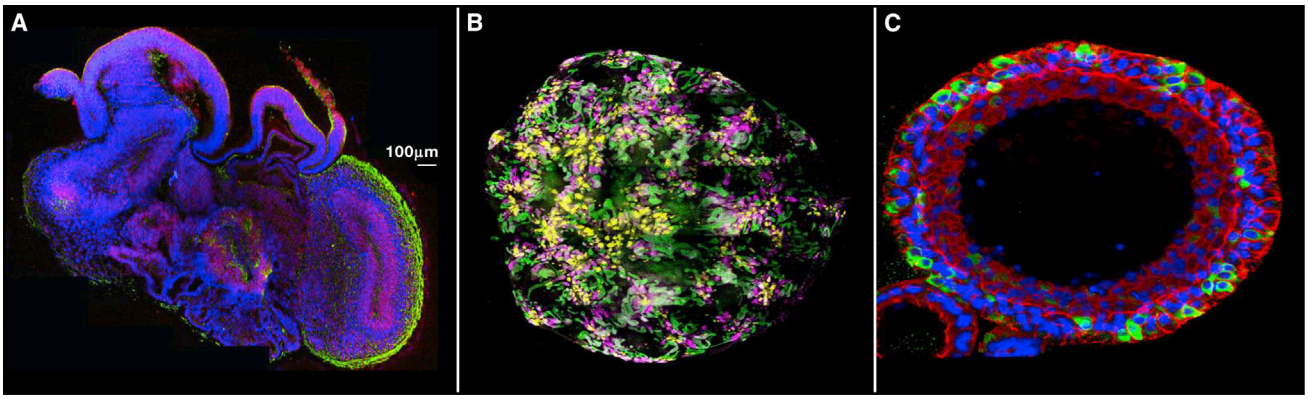

Figure 4. A "Mini-Brain" Generated from PSCs

(A) A complex morphology with heterogeneous regions containing neural progenitors (SOX2, red) and neurons (TUJ1, green) is apparent (Lancaster et al., 2013). Courtesy of Madeline Lancaster.

(B) Immunofluorescent image of an entire kidney organoid grown from PSCs with patterned nephrons. Podocytes of the forming glomeruli (NPHS1, yellow), early proximal tubules (lotus tetragonolobus lectin, pink), and distal tubules/collecting ducts (E-Cadherin, green). Courtesy of Melissa Little.

(C) 3D reconstruction of the midsection of a human aSC-derived lung organoid stained for intermediate filaments of basal cells (green), the actin cytoskeleton (red), and nuclei (blue) and imaged by confocal microscopy (N. Sachs and H.C., unpublished data).

xerostomia, this condition may present an early opportunity for the development of organoid technology-based cell therapy.

Esophagus

All examples above represent simple or two-layered epithelia. Lagasse and colleagues showed that the keratinizing stratified epithelium of the esophagus can also be cultured as organoids in "mini-gut" medium (DeWard et al., 2014). Basal cells in the mouse esophagus represent a heterogeneous population of proliferative cells. When plated as single cells, these give rise to organoids that were morphologically similar to normal esophageal tissue, with small basal-like cells in contact with the extracellular matrix, large flat suprabasal-like cells in the interior, and hardened keratinized material in the center. Expression of specific markers for each of these cell types confirmed the correct layering of the organoid walls. It will be of interest to determine whether basal cells from other squamous epithelia (epidermis, vagina) will also be amenable to organoid culture.

Applications of Organoid Technology

Both PCS- and aSC-based organoids can be initiated from single cells and cultured long-term and are amenable to essentially all cell-biological and molecular analyses that have been developed for "traditional" cell lines. As such, they provide a new window—between cell lines and in vivo studies—to studying basic gene functions and cellular processes. In addition to this, organoid technology also holds great promise for translational research. Below, I give some examples of its translational applications.

Infectious Disease

Since organoids—unlike cell lines—ideally represent all cellular components of a given organ, they are theoretically well suited for infectious disease studies, particularly of pathogens that are restricted to man and are dependent on specialized cell types. In an illustrative application, iPS-derived lung organoids were generated from an otherwise healthy child who suffered life-threatening influenza and carried null alleles in the interferon regulatory factor 7 gene. These organoids produced less type I interferon and displayed increased influenza virus replication (Ciancanelli et al., 2015). In another example, human stomach organoids, grown from PSCs or aSCs, can be productively infected by *Helicobacter pylori* (Bartfeld et al., 2015; McCracken et al., 2014).

As a striking example, Qian et al. developed a miniaturized spinning bioreactor to generate forebrain-specific organoids from human iPSCs, following the Lancaster/Knoblich protocol. These organoids recapitulate many features of cortical development, including the formation of a distinct human-specific outer radial glia cell layer. Infection of these developing forebrain organoids with Zika virus (ZIKV) resulted in the preferential infection of neural progenitors, resulting in cell death, decreased proliferation, and a reduced neuronal cell-layer volume, thus modeling ZIKV-associated microcephaly. The authors propose this as a versatile experimental for mechanistic studies as well as for testing of potential ZIKV antiviral drugs (Qian et al., 2016).

Hereditary Disease

Organoids can be used to study and model organ-specific monogenic hereditary diseases. Knoblich and colleagues identified a patient with a mutation in the CDK5RAP2 and severe microcephaly. The corresponding iPS cells made significant smaller "mini-brains," containing only occasional neuroepithelial regions with signs of remature neural differentiation, a phenotype that could be rescued by reintroducing the CDK5RAP2 protein (Lancaster et al., 2013).

Cystic fibrosis (CF) is caused by a spectrum of mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel that is normally expressed in epithelial cells of many organs. Mirroring the in vivo situation, surface expression of CFTR was absent in iPS-derived lung organoids from CF patients but could be restored by treatment with a (then still experimental) small molecule that corrects some of the common CF-processing mutations (Wong et al., 2012). Dekkers and

colleagues derived intestinal organoids from rectal biopsies of a series of CF patients. Forskolin induces a robust swelling of wild-type organoids due to fluid transport to the organoid lumen. This swelling response is absent in CF organoids yet can be restored for the common, temperature-sensitive CFTR-F508del mutant by culturing at 27°C and also by the addition of experimental CFTR corrector compounds (Dekkers et al., 2013).

Independently, the Verma lab generated iPS cells from CF patients and corrected the mutation by CRISPR/Cas9. The corrected iPS cells were subsequently converted to mature airway epithelial cells demonstrating recovery of normal CFTR function (Firth et al., 2015).

Liver organoids from alpha 1-antitrypsin deficiency patients reproduced the deleterious effects of the mutant protein precipitates in hepatocytes, while the absence of mature biliary cells in liver organoids from an Alagille syndrome patient mirrored the in vivo biliary tree abnormalities (Huch et al., 2015). Liver organoids from dogs deficient in the copper-transporter COMMD1 mimicked the disease by accumulating toxic levels of copper, which could be salvaged by re-expression of wild-type COMMD1 protein (Nantasanti et al., 2015).

Toxicology

The possibility to grow human organoids representative of the main targets for drug-related toxicity (gut, liver, kidney) opens up theoretical avenues to complement animal-based toxicology with assays performed directly on these vulnerable human tissues. In one such example, Little and colleagues have utilized human kidney organoids to illustrate that cisplatin acts as a nephrotoxicant (Takasato et al., 2015).

Cancer

Once culturing protocols for human aSC-based organoids were established, we have shown the feasibility of growing organoids from primary colon, prostate, and pancreatic cancers (Boj et al., 2015; Gao et al., 2014; Sato et al., 2011; van de Wetering et al., 2015). These cancer organoids provide the unique opportunity for functional testing (e.g., for drug sensitivity) and for correlating such data with the genetic make-up of individual tumors.

Cancer can also be modeled in organoids derived from wild-type stem cells. Kuo and colleagues probed the metastatic potential of TGFBR2 loss in murine stomach organoids by its shRNA knockdown within *Cdh1*$^{-/-}$;*Tp53*$^{-/-}$ organoids. This resulted in invasive phenotypes in vitro and in robust metastasis in vivo (Nadauld et al., 2014). Using similar shRNA-based strategies, the same authors created combinatorial Apc, p53, KrasG12D, and Smad4 mutations in wild-type murine colon organoids and observed progressive transformation to an invasive adenocarcinoma-like histology in vivo, recapitulating the multi-hit model of colorectal cancer (CRC) (Li et al., 2014). In a different approach, Huang et al. established a three-step culture method using human PSCs to generate 3D structures closely resembling human fetal exocrine pancreas (Huang et al., 2015). Expression of mutant *KRAS* and/or *TP53* in these early pancreas organoids induced abnormal ductal architecture and nuclear morphology consistent with neoplastic transformation in culture and in vivo. 17 of 20 primary human pancreas cancers could be propagated under the "third-step" conditions (i.e., EGF and FGF). Thus, more than one culture condition can be applied to efficiently grow human pancreas cancer organoids (Boj et al., 2015; Huang et al., 2015).

In a marriage between organoid and CRISPR/CAS9 technologies, two independent studies have modeled the "adenoma-carcinoma sequence" by introducing four sequential mutations into human colon organoid stem cells. Xenotransplantation revealed the progressive transformation of the wild-type stem cells into adenocarcinomas (Drost et al., 2015; Matano et al., 2015). These approaches may allow rapid modeling of novel (combinations of) gene mutations, as found in the ongoing genome- and world-wide sequencing efforts on large solid tumor panels.

Personalized Medicine

In principle, the aSC-based organoid technology allows rapid ex vivo testing of drug responses on the affected tissue of individual patients. As a first example, the colon organoid-based CF test (Dekkers et al., 2013) can be read out in weeks after biopsy. The approach has already been applied for identification and successful treatment of patients with very rare CFTR mutations, who otherwise have no access to the recently introduced CF drugs (Dekkers, 2016). The feasibility of culturing various solid tumors directly from the patient in the form of tumor organoids (see above) holds a similar promise, yet the applicability of such an approach is less clear than in the case of the "single genetic lesion" CF organoids. Tumor organoids grow with unpredictable and often slower kinetics when compared to wild-type organoids, and—like the original tumors—display a heterogeneous genetic make-up. Ongoing trials will reveal the validity and applicability of tumor organoids in the assessment of drug response at the level of the individual patient.

Regenerative Medicine and Gene Therapy

Proof-of-concept studies have demonstrated the feasibility of expanding organoids from (single) aSCs followed by safe transplantation into animals. This was first done for murine small intestine (Fordham et al., 2013; Yui et al., 2012). When small intestinal organoids are transplanted to colon, they retain original small intestinal features like villus formation and the presence of Paneth cells, indicative of the phenotypic stability of cultured aSC organoids (Fukuda et al., 2014). Of note, it may be advantageous to modify the culture conditions to selectively expand stem cells at the cost of differentiated cells (Wang et al., 2015; Yin et al., 2014) prior to transplantation. We have used CRISPR/Cas9 genome editing to correct the CFTR locus by homologous recombination in cultured intestinal stem cells of CF patients. The corrected allele was fully functional, as demonstrated in clonally expanded organoids (Schwank et al., 2013). This approach can presumably be used for gene correction in any clonally expandable cell population derived from monogenic hereditary disease patients.

Conclusions

In this Review, I have attempted to describe the state of the art of the explosively developing field of PSC- and aSC-based organoids. The current versions of organoids have clear limitations, e.g., innervation, blood vessels, and immune cells are absent, and as a consequence, disease processes are only partially recapitulated. Yet it is anticipated that the potent self-organizing

properties of organoids may extend beyond their current boundaries and allow the proper incorporation of additional cellular (or microbial) elements. From a basic science perspective, PSC-based organoids will by their very nature play a key role in understanding the developmental biology of organs and will thus complement the long tradition of in vivo studies in this field. From the same perspective, aSC-based organoids provide basic insights into the processes that allow aSCs to maintain and repair established tissues. Yet, because of the ease of production and the close resemblance to human organs in health and disease, organoids hold great appeal for translational research and invite an almost immediate application into the clinic.

ACKNOWLEDGMENTS

Thanks for comments to the text to Melissa Little, Hans Snoeck, Juergen Knoblich, and Esther Verheyen and to Janny van Eldik for secretarial help. Apologies to those scientists whose work could not be cited due to space restrictions. Written as visiting professor at Memorial Sloan Kettering Cancer Center, New York, NY. H.C. is named as inventor on several patents related to Lgr5 stem-cell-based organoid technology.

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