Organoid Research Techniques

Evolution and Applications

Cover Image: STEMCELL Technologies

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INTRODUCTION

> *"I heard about organoids and thought they might be the perfect model to study all the processes that I'm interested in," says Tamara Zietek, Principal Investigator in the Nutrition Physiology Laboratory at the Technical University of Munich. Before using organoids, Zietek's group worked both with an in vivo—mice—and in vitro—cell lines—system to answer their research questions, but they were on the hunt for a system that combined the advantages of both. Using primary cell cultures was an improvement, but because the cells couldn't be passaged, it was not a long-term system. So, Zietek used organoid model systems to better understand intestinal nutrient transport and sensing.*

In recent years, our cumulative understanding of organ physiology, development, and maintenance has resulted in the creation of three-dimensional (3D) organoids—cultured 3D cell structures that model features of organ function, composition, and development. Given that the organs of our body occupy 3D space, organoids better represent the physiological system than their two-dimensional culture counterparts for the purpose of organ studies as they recapitulate the signaling and morphological cues that can occur within the human body. Scientists have thus turned to using organoids to study normal and pathological conditions, and as a method to test potential therapeutics for human disease.

The methods for culturing organoids are tissue-dependent, but some overall principles apply. A scientist embeds the starting material—such as progenitor cells derived from pluripotent stem cells (PSCs) or adult stem cells (ASCs) harvested from tissue samples—in an extracellular matrix. The cells are maintained in culture media containing the nutrients and growth factors necessary to mimic the *in vivo* cellular environment. Under these conditions, the starting cells expand and self-organize to build a

3D structure—the organoids—which can be maintained for long periods of time. For some epithelial organoids (e.g., intestinal organoids), the cultures can be maintained indefinitely through routine passaging (i.e., breaking the organoids into small sections and reseeding them in new cultures).

Developing culture conditions for organoids is not a trivial matter. Certain conditions must be met for the organ-resident stem cells to be maintained and differentiated into the appropriate complement of cells. This fundamental property of organoid generation makes the system ideal for understanding organ development and the biology of the adult- or tissue-resident stem cells that maintain a given tissue throughout life. Organoids also lend themselves to many other applications, such as cell biology, drug development, and the understanding of disease. Their wide range of applications, coupled with their potential to significantly reduce the use of animal models while allowing for facile experimentation on human cells, makes organoids valuable model systems. Consequently, organoid model systems are seeing rapid adoption in laboratories world-wide.
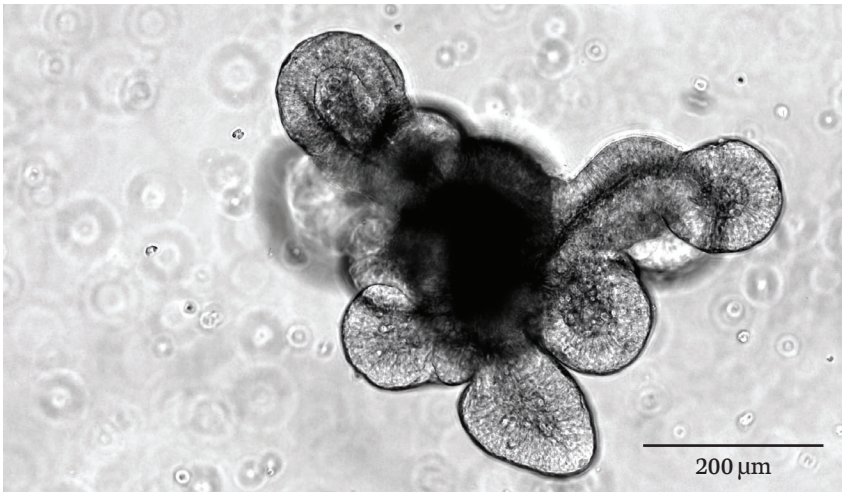
Figure 1. Light microscope visualization of a mouse intestinal organoid.
Source: STEMCELL Technologies

Organoids can be classified in a variety of ways, depending on the starting cells used, the tissue, and whether cells are from healthy or diseased sources. To date, organoids have been successfully generated from a wide variety of tissues, including brain, breast, intestine, kidney, liver, lung, optic cup, pancreas, and prostate. Additionally, organoids can be generated from tumor tissue, resulting in organoid cultures that closely model the *in vivo* tumor characteristics and genetic heterogeneity—something that has not been possible in 2D cultures.

Organoids do represent a significant leap in understanding stem cell biology and organ development, but combinatorial and/or complementary techniques could drive even more applications. Therefore, combining organoid generation with evolving techniques, such as the gene-editing technique CRISPR-Cas9, might prove vital to more thoroughly investigating biological and medical questions.

HISTORIC MILESTONES

The major achievements in research on and with organoids can be explored in this chronological list of key publications:

- Ootani, A., Li, X., Sangiorgi, E., *et al.* (2009). Sustained *in vitro* intestinal epithelial culture within a Wnt-dependent stem cell niche. *Nat. Med.* 15(6):701–706.

  ~In search of a culture system that mimics the growth and differentiation of the intestine, Calvin Kuo—Maureen Lyles D'Ambrogio Professor of Medicine at Stanford University—and his colleagues developed a method for creating intestinal organoids. They also showed that an antagonist of the Wnt growth factor inhibited growth of the organoids, and that other treatments could be used to trigger the differentiation of specific types of cells, such as goblet and enteroendocrine cells.

- Sato, T., Vries, R.G., Snippert, H.J., *et al.* (2009). Single Lgr5 stem cells build crypt-villus structures *in vitro* without a mesenchymal niche. *Nat.* 459(7244):262–265.

  ~New cells in the intestinal wall arise in the crypts, which are the dips between villi. Toshiro Sato of the Keio University School of Medicine—then working in the lab of Hans Clevers at the University Medical Center Utrecht and Utrecht University—and his colleagues developed a system in which crypt cells could be turned into organoids. In particular, the scientists started these organoids with crypt cells expressing *Lgr5*, which triggers cycling in these cells.

- Sato, T., Stange, D.E., Ferrante, M., *et al.* (2011). Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology* 141(5):1762–1772.

  ~Continuing the work with intestinal organoids created from crypt-derived stem cells, Sato and his colleagues developed methods to grow

healthy and disease-specific organoids from mouse colon and human small intestine and colon. After optimizing methods for culturing these organoids, Sato's team reported: "We developed a technology that can be used to study infected, inflammatory, or neoplastic tissues from the human gastrointestinal tract." The researchers added that they found no "inherent restriction in the replicative potential of adult stem cells … *ex vivo*."

- Spence, J.R., Mayhew, C.N., Rankin, S.A. (2011). Directed differentiation of human pluripotent stem cells into intestinal tissue *in vitro*. *Nat.* 470(7332):105–109.

  ~Using human PSCs, Jason Spence—then at Cincinnati Children's Hospital Medical Center and now associate professor of internal medicine at the University of Michigan Medical School—and his colleagues developed what they described as "a robust and efficient process to direct the differentiation of human PSCs into intestinal tissue *in vitro* using a temporal series of growth factor manipulations to mimic embryonic intestinal development." This involved endoderm formation and patterning, hindgut specification and morphogenesis, plus a culture system for intestinal growth, morphogenesis, and cytodifferentiation. "The resulting three-dimensional intestinal 'organoids' consisted of a polarized, columnar epithelium that was patterned into villus-like structures and crypt-like proliferative zones that expressed intestinal stem cell markers," Spence's team noted.

- Kadoshima, T., Sakaguchi, H., Nakano, T., *et al.* (2013). Self-organization of axial polarity, inside-out layer pattern, and species-specific progenitor dynamics in human ES cell-derived neocortex. *Proc. Natl. Acad. Sci. USA* 110(50):20284–20289.

  ~Working with cortical neuroepithelium derived from human embryonic stem cells, Taisuke Kadoshima—then at the RIKEN Center for Developmental Biology and now at Asubio Pharma—and his collaborators studied neocorticogenesis in neural organoids.

"Self-organized cortical tissue spontaneously forms a polarity along the dorsocaudal-ventrorostral axis and undergoes region-specific rolling morphogenesis that generates a semispherical structure," the researchers wrote. "The neuroepithelium self-forms a multilayered structure including three neuronal zones (subplate, cortical plate, and Cajal-Retzius cell zones) and three progenitor zones (ventricular, subventricular, and intermediate) in the same apical-basal order as seen in the human fetal cortex in the early second trimester." Based on these and other features, the scientists concluded that "human neocorticogenesis involves intrinsic programs that enable the emergence of complex neocortical features."

- Lancaster, M.A., Renner, M., Martin, C.A., *et al.* (2013). Cerebral organoids model human brain development and microcephaly. *Nat.* 501(7467):373–379.

  ~"The complexity of the human brain has made it difficult to study many brain disorders in model organisms, highlighting the need for an *in vitro* model of human brain development," wrote Madeline Lancaster—then at the Institute of Molecular Biotechnology of the Austrian Academy of Science and now a principal investigator at the Medical Research Council (MRC) Laboratory of Molecular Biology— and her coworkers. Cerebral organoids were generated using human-derived PSCs in healthy controls as well as microcephalic patients. In addition to forming regions like the cerebral cortex, these organoids "recapitulate features of human cortical development, namely characteristic progenitor zone organization with abundant outer radial glial stem cells," Lancaster's team pointed out.

- Bagley, J.A., Reumann, D., Bian, S., *et al.* (2017). Fused cerebral organoids model interactions between brain regions. *Nat. Methods* 15:734–751.

  ~To build a neural organoid that allows more advanced studies of the brain, the 3D structure needs some representation of brain regions. To move toward such a neural organoid, Joshua Bagley—a postdoctoral

researcher in Juergen Knoblich's lab at the Institute of Molecular Bio-technology of the Austrian Academy of Science in Vienna, Austria—and his colleagues used small molecule patterning to create dorsal- and ventral-brain organoids from human induced PSCs (iPSCs) and fused them. These structures exhibited some of the developmental processes found in human embryonic brains, including γ-aminobutyric acid (GABA)-producing interneurons developed in the ventral portion of the brain and migrating to the dorsal region. Researchers showed the migrating cells were neurons as indicated by the expression of HuC/D, and mature by the expression of MAP2. Based on other molecular markers, the scientists reported: "Our results indicate that organoid fusions contain diverse interneuron subtypes originating from the major ventral forebrain subregions." By producing organoids that mimic other brain regions and fusing them, this methodology can be used to study a wide range of neuronal circuits.

- Birey, F., Andersen, J., Makinson, C.D., *et al.* (2017). Assembly of functionally integrated human forebrain spheroids. Nat. 545:54–59.

~ The dorsal forebrain, also known as the pallium, contains excitatory neurons that release the neurotransmitter glutamate. The ventral forebrain, or subpallium, contains inhibitory neurons that release GABA. To study the development of neural circuits in the cortex, Fikri Birey—a postdoctoral researcher in Sergiu Paşca's lab at Stanford University—and his colleagues created human cortical (dorsal) spheroids (hCS) and human subpallium spheroids (hSS) from hPSCs. Placing one of each kind of spheroid in a tube, they combined in three days. Using green fluorescent protein (GFP), the scientists showed cells moving from the hSS to the hCS. The team used this model to study Timothy syndrome (TS), in which defects in cortical-neuron migration result in various neural deficits, including autism spectrum disorders. The scientists made hCS and hSS from patients with TS. Compared to hCS-hSS structures from control subjects, neurons migrated more slowly in the structures from people with TS. Studies in rodent models have suggested that the migration of L-type calcium

channels (LTCCs) play some part in TS. Birey's team showed "that the migration defect in interneurons carrying the TS gain-of-function mutation can be restored by reducing the activity of LTCCs." The cortical excitatory-inhibitory balance in patient-derived models of autism can now be studied mechanistically in vitro. This work demonstrated how anatomical and molecular aspects of neurodevelopment, even in the cortex, can be explored with organoids.

- Freedman, B.S., Brooks, C.R., Lam, A.Q., *et al.* (2015). Modelling kidney disease with CRISPR-mutant kidney organoids derived from human pluripotent epiblast spheroids. *Nat. Commun.* 6:8715.

  ~Benjamin Freedman—then at Harvard Medical School and now at the University of Washington School of Medicine—and his coauthors wanted to know if human PSC (hPSC)-derived kidney cells could "reconstitute tissue-specific phenotypes." To find out, the researchers made 3D cultures of epiblast-stage hPSCs and showed that inhibiting an enzyme—glycogen synthase kinase 3 beta (GSK3β)—caused the spheroids to differentiate "into segmented, nephron-like kidney organoids containing cell populations with characteristics of proximal tubules, podocytes and endothelium." In addition, these scientists showed that gene manipulation could be used to create models of health conditions. For example, the authors reported: "Knockout of the polycystic kidney disease genes *PKD1* or *PKD2* induces cyst formation from kidney tubules."

- Takasato, M., Er, P.X., Chiu, H.S., *et al.* (2015). Kidney organoids from human iPS cells contain multiple lineages and model human nephrogenesis. *Nat.* 526(7574):564–568.

  ~The kidney portrays the diversity of cell types that can be required to build an organoid that reliably replicates the actual structure. "Regenerating the kidney requires the induction of the more than 20 distinct cell types required for excretion and the regulation of pH, and electrolyte and fluid balance," wrote Minoru Takasato—then at the

University of Queensland and now at RIKEN Center for Biosystems Dynamics Research—and a team of scientists. These cells build specific parts of the kidney, such as the collecting duct and nephrons. In this article, Takasato and his colleagues described a technique to generate kidney organoids that included these structures. As the researcher wrote: "Within these organoids, individual nephrons segment into distal and proximal tubules, early loops of Henle, and glomeruli containing podocytes elaborating foot processes and undergoing vascularization." The researcher concluded that these kidney organoids could be used for future applications, including nephrotoxicity screening, disease modelling and as a source of cells for therapy."

IN PRACTICE

Organoid Types and Culture Conditions

There are two primary classifications of organoid systems: organoids cultured from PSCs and organoids cultured from adult stem or progenitor cells. PSC-derived organoids are generated by differentiation of PSCs to lineage-specific progenitor cells that form the desired organoid. Organoid formation is then achieved by culturing these progenitors in conditions that mimic the *in vivo* developmental conditions of that organ. For brain organoids, by omitting or adding patterning factors, the organoid can either be allowed to self-organize into an organoid with several different brain regions (e.g., cerebral organoids) or can be directed to form an organoid with specific brain regions (e.g., forebrain or midbrain organoids). The advantage of PSC-derived organoids is their easy-to-access starting material, because previously-established or patient-specific PSC lines can be used. These organoids tend to model the developing, rather than the adult organ. ASC-derived organoids are cultured from tissue-specific stem or progenitor cells that are responsible for maintenance of a given organ *in vivo*. The organoids grown from these progenitors tend to more closely model adult tissue and have the advantage that they can recapitulate both congenital and non-congenital disease states, including modeling epigenetic and tumor signatures.

Both classes of organoids are cultured in specific cell-culture media within an extracellular matrix that is required to support the 3D structure. The cell culture medium used depends on the type and tissue of the organoid, and in general mimics the signaling environment the cultured cells would experience *in vivo*. For PSC-derived cerebral organoids, for example, the culture conditions mimic the signaling present in the developing brain, allowing the neural progenitors present in early stages of the organoid to differentiate and self-organize into the laminar structure of the cerebrum. In similar fashion, the culture conditions for ASC-derived intestinal organoids mimic the signaling present at the intestinal crypt base, the stem cell

niche of intestinal stem cells. This allows for self-renewal of the stem cell population as well as differentiation of a subset of this population to form the cellular complement of the intestine.

Adult Stem Cell–Derived Organoids

Sato *et al.* (2009) developed a method for culturing ASC-derived intestinal organoids from isolated intestinal crypts. This kicked off a decade of innovation in organotypic 3D cell culture techniques. The culture model of these intestinal organoids exploits the biology of the intestinal stem cells, which *in vivo* actively divide throughout life to replenish their own population and turnover the entire intestinal epithelium every 5–10 days. The environmental conditions that enable these replenishing properties *in vivo* are recapitulated in the organoid culture system. This allows the same cells to create a faithful intestinal model *in vitro*. Although not all tissues have ASCs with the same regenerative capacity during homeostasis, many organs contain a population of cells that carry the ability to regenerate tissue under specific conditions. This has enabled development of organoid culture conditions for a wide variety of tissues, including liver, pancreas, prostate, breast, and airway.

Generating organoids from ASCs has several advantages. First, because the cultures are generated from phenotypically adult cells, the resultant cultures also tend to exhibit an adult phenotype. The protocols also tend to be shorter than those needed to generate PSC-derived organoids, because the starting material is already a tissue-specific progenitor. The techniques are comparatively easy to learn and adopt, allowing labs to add organoid experiments to their already established laboratory repertoire. Once established, many ASC-derived organoid cultures can be maintained long-term through routine passaging and can be cryopreserved; however, the original starting material requires patient biopsy and associated consent that can significantly limit access for many researchers, especially industrial laboratories.

While there is substantial overlap in the applications of ASC- and PSC-derived organoids, ASC-derived organoids are uniquely suited to some

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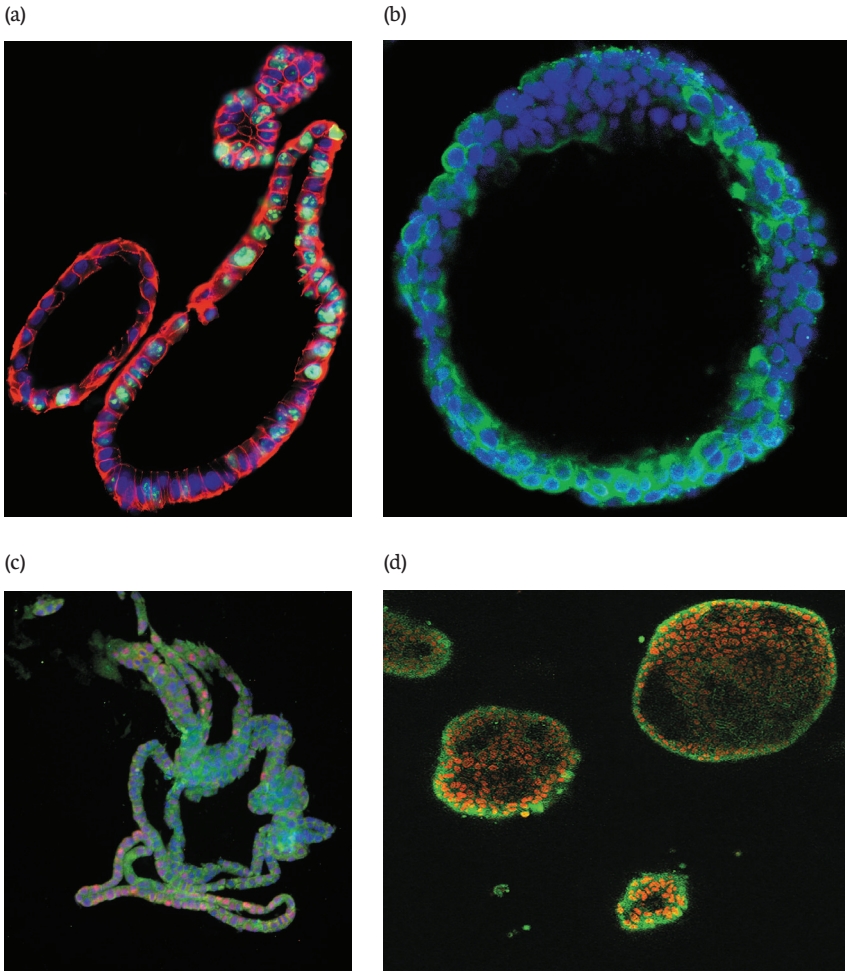
Figure 2. Adult stem cell-derived organoids: (a) Human colonic organoids; (b) mouse prostate organoids; (c) pancreatic exocrine organoids; and (d) hepatic progenitor organoids.

Source: STEMCELL Technologies

specific applications. For example, ASC-derived organoids can be used to model non-genetic pathologies and cancers, both of which are challenging for a PSC-derived system. Indeed, tumor organoids derived from ASCs are rapidly gaining recognition for their utility in patient-specific tumor models. The cancer characteristics, genetic heterogeneity, and drug response profile are maintained with high fidelity in the organoids, allowing for precision-medicine applications.

The source of the adult stem cells is critical to the successful generation and use of organoids. Researchers often use mouse organoids to compare results from *in vivo* and *in vitro* studies. Organoids made from human stem cells, however, allow unique uses, such as studying therapeutic impact on a patient's own tumor (precision medicine) or the basic biology of human stem cells.

As with any emerging technology, organoids must be validated as appropriate model systems for any given application. As an example, Zietek *et al.* (2015) created intestinal organoids from wild-type mice and ones lacking specific nutrient transporters. The results showed that the structures serve as excellent models for research because they maintain the phenotypic and functional features of mouse intestines. Likewise, ASC-derived organoids are being explored and adopted for a wide range of applications ranging from basic, academic research to drug development.

Adult Stem Cell Case Studies

The epithelium in the mucosal surfaces of the intestines is replaced every 5–7 days. Stem cells in the crypts are the source of this self-renewal, and these cells can be stimulated to differentiate into any of the cell types that make up the lining of the intestine. To explore the molecular details of those mechanisms, Sehgal *et al.* (2018) used Gentle Cell Dissociation Reagent to dissociate crypts from mouse small intestine, and then cultured the crypts with IntestiCult™Organoid Growth Medium (Mouse) to make enteroids. Scientists already knew that colony-stimulating factor 1 (CSF1) plays a role in maintaining gut epithelium, but the mechanism was unknown.

CSF1 drives the differentiation of macrophages from monocytes. Through macrophage ablation and CSF1R blockade studies, the scientists showed that CSF1-dependent macrophages "influence intestinal epithelial differentiation and homeostasis" by aiding in the differentiation of intestinal epithelial Paneth cells, which release antimicrobials that block bacterial infection in the crypts and prevent bacteria from crossing the intestinal
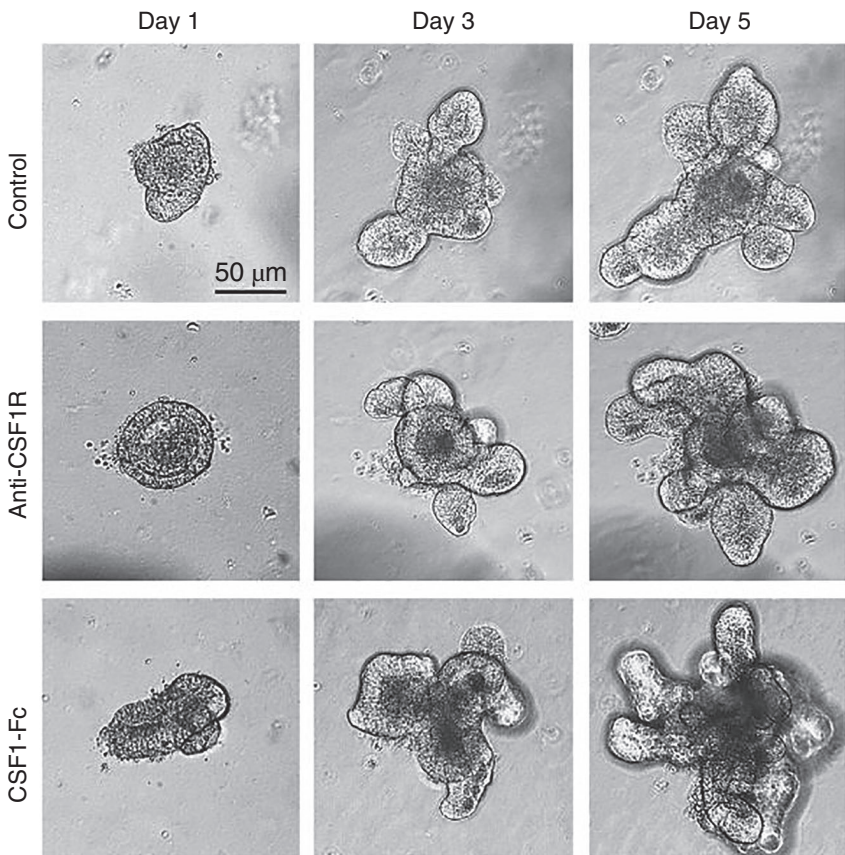
Figure 3. No obvious morphological changes distinguish mouse intestinal organoids treated with anti-CSF1R or CSF1.

epithelium. This work yields two general conclusions: 1) the utility of organoids allowed quick assessment of the role of CSF1 in a physiologically relevant *in vitro* cell culture model; and 2) this work provided further understanding of CSF1R-dependent, crypt-associated macrophages that are "constitutively required to maintain the intestinal stem cell niche in the small intestine."

Another example of the utility of organoids can be found in studies of the liver. Broutier *et al.* (2017) described generating tumor organoids from liver tumor tissue from patients who had not been treated for primary liver cancer (PLC). With this technique, the researchers generated organoids from the three most common subtypes of PLC: hepatocellular carcinoma (HCC), cholangiocarcinoma (CC), and combined HCC/CC (CHC) tumors.

Several comparisons confirmed that the organoids mimic the original tumor. Histologically, the tumoroids maintained patient-specific heterogenous morphological features of each tumor subtype. For instance, HCC and CHC tumoroids included compact structures and CC tumoroids included irregular cyst structures—all different than the ordered, cyst-like structures in healthy liver organoids. Based on genome-wide transcriptomic analysis (RNA sequencing, or RNAseq) and comparison to the known gene-expression patterns for the subtypes of PLC, Broutier *et al.* reported: "Gene expression correlation analysis indicated that each tumoroid line correlated to its corresponding tissue-of-origin, but not with the other subtypes."

Broutier *et al.* used these organoids to identify an inhibitor of the extracellular-signal-regulated kinase (ERK) pathway that might lead to a PLC treatment. As the researchers concluded, this work demonstrates "the wide-ranging biomedical utilities of PLC-derived organoid models in furthering the understanding of liver cancer biology and in developing personalized-medicine approaches for the disease."

Pluripotent Stem Cell-Derived Organoids

As noted above, organoids can also be created from PSCs. Here, the starting cells can be established PSC cell lines or patient-specific induced PSCs (iPSCs). The capacity of PSCs to differentiate into any cell type of the
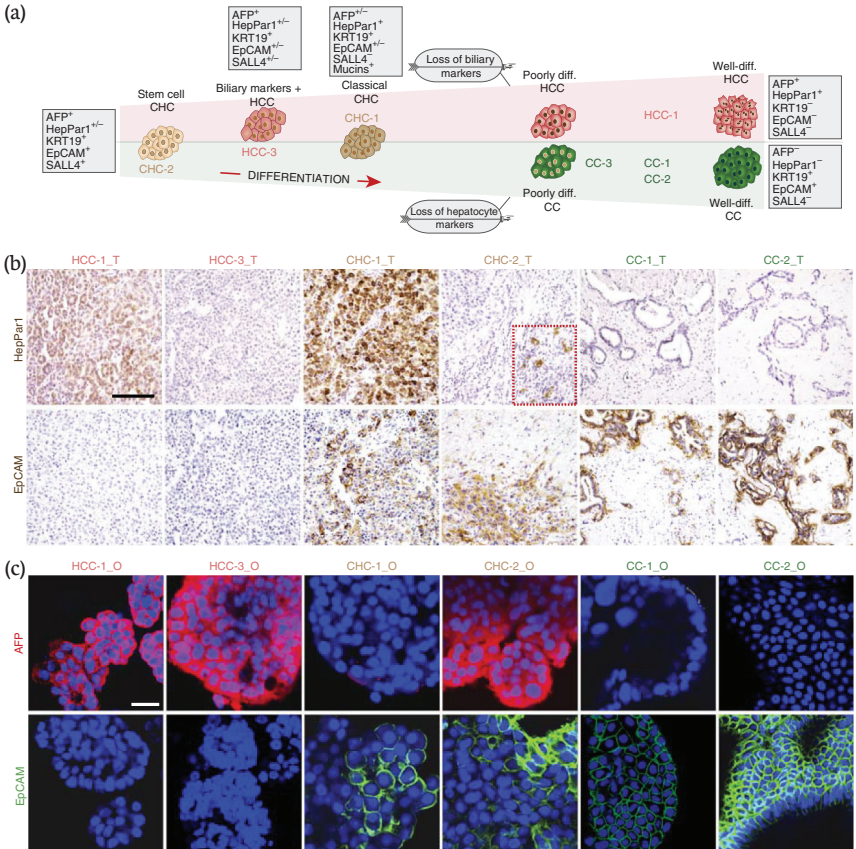
Figure 4. Human PLC tumoroids maintain the expression patterns of the original tissue in long-term culture. (a) HCC and CC subtypes of PLC arise from distinct pathways. (b) Immunohistochemistry assays for the hepatocyte/HCC (HepPar1) and ductal/CC (EpCAM) markers on subtypes of PLC tissue. (Scale bar, 125 μm; dashed red square indicates focal staining.) (c) On tumoroids cultured for at least three months, immunofluorescence analysis shows presence of the HCC marker AFP (red) and the ductal/CC marker EpCAM (green). (Nuclei were counterstained with Hoechst33342 (blue); Scale bar, 30 μm.)

body, combined with their ability to proliferate indefinitely, make PSCs a convenient starting material for organoid culture.

PSC-derived organoid culture systems have been developed to model tissues derived from all three germ layers: endoderm, ectoderm, and

mesoderm. These systems mimic the biology of the tissues they model; for example, cerebral organoids recapitulate the developmental processes and organization of the developing human brain. Such organoids can be generated and maintained with the STEMdiff™ Cerebral Organoid Kit. Like the cerebral tissue these organoids model, the cells within the organoids can be maintained for weeks and months, if they have access to the right nutrients, but cannot be used to initiate new organoid cultures. This is similar to the *in vivo* brain that contains proliferative progenitors during development and loses the vast majority of its ability to generate new neurons in adulthood. This contrasts with the epithelial component of some organs, such as the intestine, which maintain an active adult stem cell population to constantly replenish the organ's cells. PSCs can be directed to these lineages and generate organs that share many of the growth properties of ASC-derived organoids. Intestinal PSC-derived organoids, for example, can be generated and maintained long-term through routine passaging with the STEMdiff™ Intestinal Organoid Kit.

Generating organoids from PSCs comes with advantages. It is relatively easy to access starting material to generate organoids, compared with obtaining patient biopsies. Making patient-specific iPSCs also allows the generation of organoids with a specific genetic code. With human cerebral organoids, starting with hPSCs enables studies not possible with any other model system, because ethical limitations severely constrain access to primary neural tissue. Building organoids from PSCs also allows for co-differentiation of multiple lineages within one culture, thus providing the opportunity to create organoids that mimic the various tissues found in an actual organ.

Despite the advantages of making organoids from PSCs, this approach also raises some challenges. For one, the scientist needs to be proficient in maintaining PSCs to ensure high-quality starting cells. The protocols for differentiating the cells to organoids can also be quite lengthy, requiring multiple steps that mimic the developmental trajectory of cells in a developing body.

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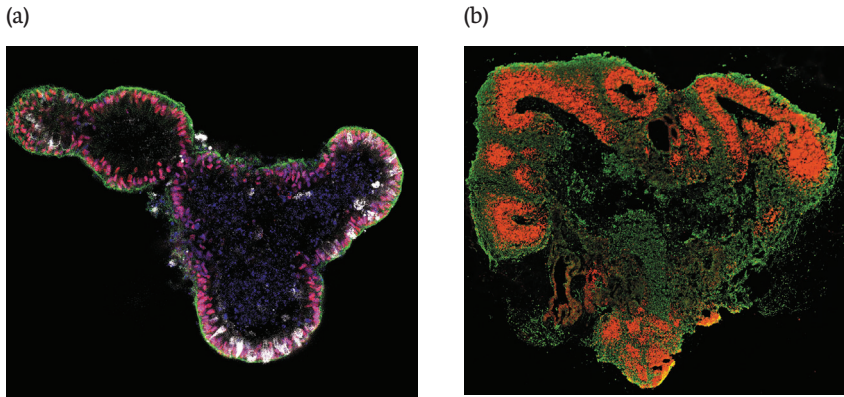
Figure 5. Pluripotent stem cell-derived organoids: (a) Human intestinal organoids; and (b) human cerebral organoids.

Source: STEMCELL Technologies

Still, organoids created from PSCs fit many applications. For intestinal organoids, using hPSCs as the starting material provides an opportunity to study developmental stages of the intestine, such as a more fetal-like organ in early passages. Genetic manipulation also provides the opportunity for other applications of PSC-derived organoids, and the cells can be genetically manipulated by adding DNA through transfection or electroporation. Also, a CRISPR-Cas9 technique can be used to edit genes within organoid systems. This method is faster than making knock-out mice. These organoid systems can also be used in combination with other technologies. For instance, information from studies of knock-out mice can be reexamined in organoids, where mechanisms can be explored in an environment that better reflects physiological conditions.

## Pluripotent Stem Cell Case Studies

Neural organoids derived from PSCs can be used in many ways, from studying normal neural development and processes to analyzing brain-based diseases. Scientists did not know, however, if neural organoids could be used to study the developmental guidance of axons involved in neural circuits. To find out, Giandomenico *et al.* (2019) generated neural organoids from human embryonic stem cells cultured with the STEMdiff™ Cerebral Organoid Kit. Mature neural organoids were combined with dissections

of mouse spinal cord, including some nearby muscle tissue, and sliced in 300-μm sections to form air-liquid interface cerebral organoids (ALI-COs). The application of pre- and post-synaptic markers showed synapses on the dendrites of mature neurons in the organoids, and Giandomenico *et al.* recorded spontaneous electrical activity using BrainPhys™ Neuronal Medium in the neurons. Moreover, injecting positive current via whole-cell patch clamping triggered action potentials, demonstrating functional maturity of the organoids.

The ALI-COs also showed many features of an actual brain. By applying RNAseq for cell types and clustering the results based on expression with principal components analysis, Giandomenico *et al.* found "a well-defined association between cell-types and their expected function suggested by the molecular profiles in each cluster." Moreover, with the ease of live imaging in the sliced culture, Giandomenico *et al.* tracked axon guidance in GFP-labelled neurons. The neurons in the ALI-COs could even drive contraction in the muscle tissue near the cord. Interestingly, contractions could be inhibited pharmacologically or by lesion of the axonal tract. This model system will enable detailed mechanistic studies of the regenerative capacity of the human nervous system.

Overall, this work shows the benefits of the ALI-CO approach, including long-term viability. The ALI-COs remained viable for up to five months, the longest time tested. Giandomenico *et al.* also noted: "These experiments are the first to our knowledge to show a functional output from a neural organoid."

The uses of PSC-derived organoids will expand as scientists combine systems to make even more accurate models of human organs. In one approach, Spence *et al.* (2011) developed a method for making human intestinal organoids. Starting with human embryonic stem cells and iPSCs, this protocol was later licensed to serve as the basis for the STEMdiff™ Intestinal Organoid Kit. This method, Spence *et al.* explained, involves a "temporal series of growth factor manipulations" to induce definitive endoderm formation, posterior endoderm patterning, hindgut specification and morphogenesis and a "pro-intestinal culture system to promote intestinal
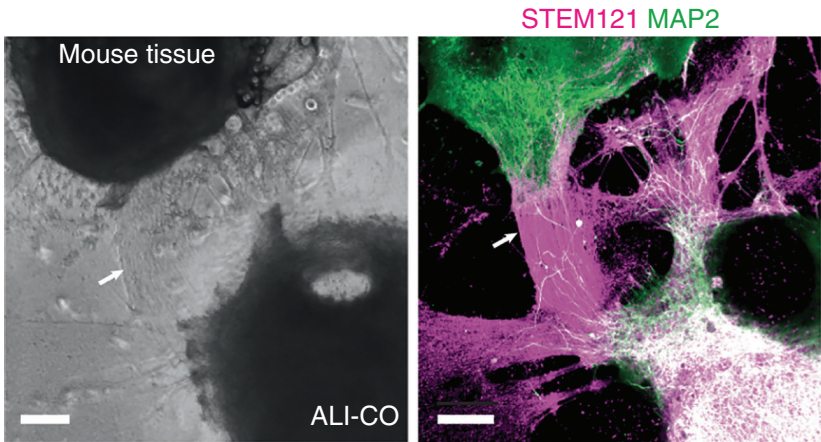
Figure 6. Brightfield image (left), shows axon tracks (arrow) in combined human ALI-CO and mouse spinal cord, plus associated tissue. Staining (right) with human-specific STEM121 (pink) shows that the tracks originate in the ALI-CO; MAP2 (green) indicates mouse neurons in the spinal cord and human neurons in the ALI-CO.

Source: Giandomenico, S.L., Mierau, S.B., Gibbons, G.M., *et al.* (2019). Cerebral organoids at the air-liquid interface generate diverse nerve tracts with functional output. *Nat. Neurosci.* 22:669–679. doi: 10.1038/s41593-019-0350-2.

growth, morphogenesis and cytodifferentiation." This is the first demonstration of a robust and efficient method of directed differentiation *in vitro* from human PSCs toward a 3D architecture and cellular composition "remarkably similar to the fetal intestine."

Workman *et al.* (2017) took this technique further to make human intestinal organoids (HIOs), which they combined with neural crest cells derived from human PSCs (hPSCs). This resulted in intestinal organoids with a functional enteric nervous system (ENS). The ENS controls the gastrointestinal tract's mobility and permeability. In the HIO+ENS organoids, Workman *et al.* used RNAseq to show transcriptional changes in the HIO+ENS versus HIO organoids. In addition, the researchers showed neuronal activity in the ENS cells, ENS-driven muscle contractions, and intestinal-like motility.

As the scientists pointed out: "Perturbations in ENS development or function are common, yet there is no human model for studying ENS-intestinal biology and disease." Workman *et al.* explored the possibility of using HIO+ENS organoids to model Hirschsprung's disease, which is
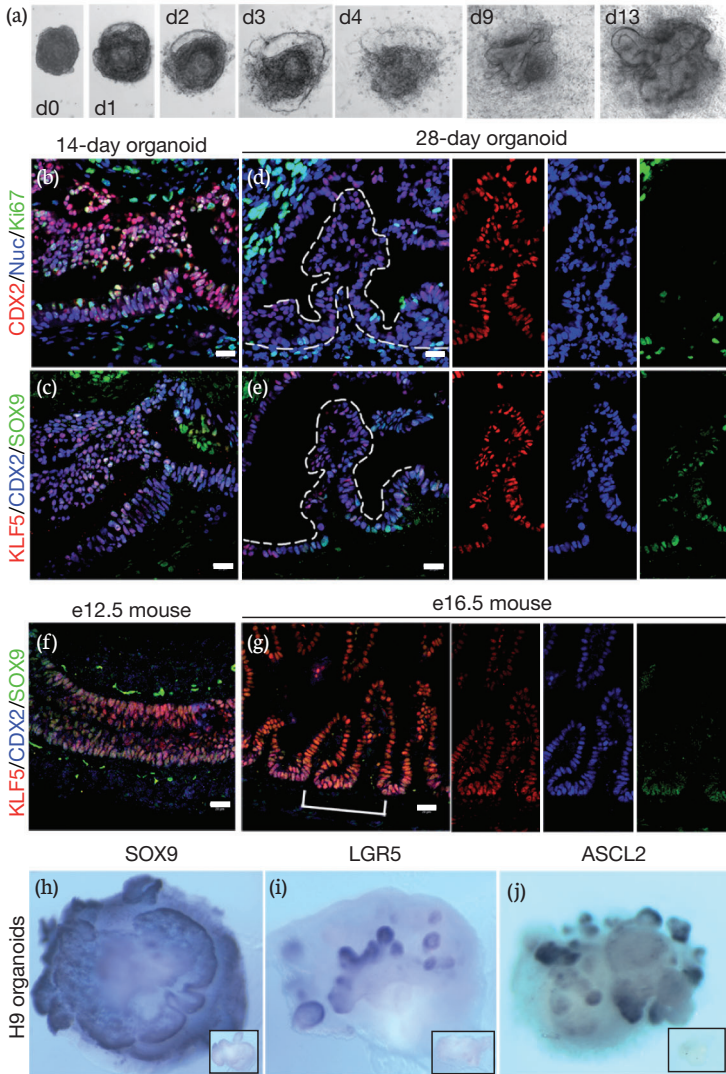
Figure 7. Human embryonic stem cells and induced pluripotent stem cells can be directed to create intestinal organoids. (a) In less than two weeks, they form highly convoluted epithelial structures surrounded by mesenchyme. (b–e) Expression of intestinal transcription factors (KLF5, CDX2, SOX9) and cell proliferation on serial sections of organoids after 14 and 28 days resemble mouse fetal intestinal development (f, g). (h, i, j) Whole mount of 56-day-old organoids show epithelial expression of Sox9 (h) and some "crypt-like" expression of the stem cell markers Lgr5 (i) and Ascl2 (j). (Insets show sense controls for each probe.)

a congenital constipation caused by a developmental defect in the ENS. Various genes have been implicated in this disease, and one is the mutated paired-like homeobox 2B (*PHOX2B*), which can cause the complete lack of neurons in the intestines of humans and mice. "Despite the obvious phenotype, the molecular pathways that are affected by *PHOX2B* mutations have not been identified in humans, so we used HIO+ENS as a model system to study this form of Hirschsprung's disease," Workman *et al.* explained.

Workman *et al.* generated hPSCs with various *PHOX2B* mutations and used them to generate HIO+ENS organoids. They concluded that "this human-PSC-derived intestinal model seems well suited for mechanistically studying genetic forms of Hirschsprung's disease in humans."
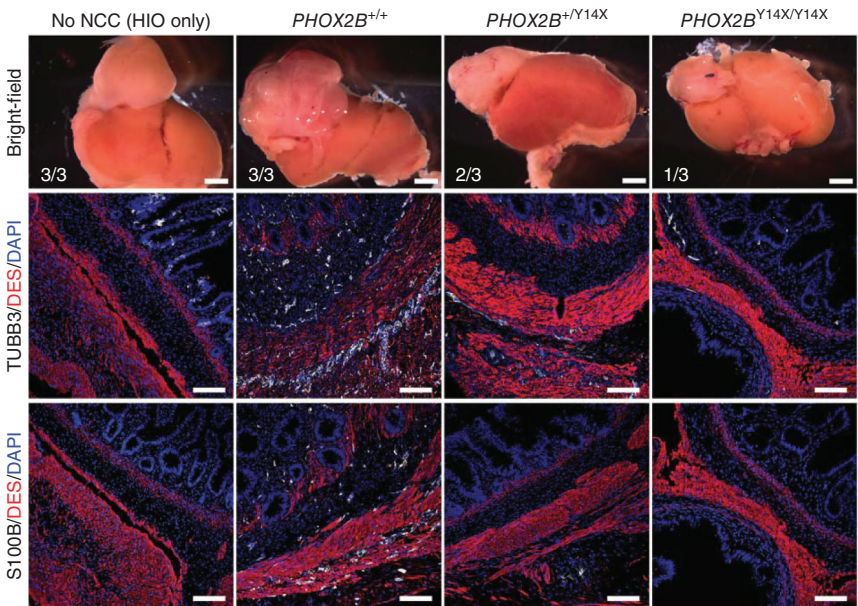
Figure 8. HIOs+ENS generated with *PHOX2B* neural crest cells. Bright-field images (top) show harvested organoids after seven weeks of post-transplantation growth with ENS carrying one of three *PHOX2B* mutations. (Fractions indicate the number of organoids that grew and contained intestinal epithelium; Scale bars, 2 mm.) TUBB3 (middle) indicates the development of neurons in the organoids, and S100β (bottom) shows the development of glia. (Scale bars, 100 μm.)

Source: Workman, M.J., Mahe, M.M., Trisno, S., *et al.* (2017). Engineered human pluripotent stem cell-derived intestinal tissues with a functional enteric nervous system. *Nature Medicine*, 23:49–59. doi: 10.1038/nm.4233.

PROBLEMS & SOLUTIONS

Standardizing culture conditions is the key challenge to using organoids in research. Some methods are inherently prone to variability and make generation of reproducible data a challenge. These problems usually arise from the medium used in culture, often including very complex components that are labor-intensive to prepare and prone to variable performance. Published media also vary in composition, which makes standardization nearly impossible. The inconsistency makes collaboration and reproducibility across labs challenging.

Using a complete commercial medium can be an effective option to manage lab resources while making it easier to compare data across experiments, users, and collaborators. To establish and maintain mouse intestinal organoids from mouse intestine cells, for example, IntestiCult™Organoid Growth Medium (Mouse) is a serum-free option. This medium also creates mouse intestinal organoids that include all the expected types of cells in the adult intestinal epithelium. Similarly, IntestiCult™Organoid Growth Medium (Human) can be used to establish and maintain human intestinal organoids. Other products are available from STEMCELL Technologies to support culture of ASC-derived organoids from a variety of tissues, including liver and pancreas.

In addition to differing media compositions, variations on differentiation protocols for stem cells can produce significantly different results, making it extremely difficult or impossible to compare data across labs using different directed differentiation protocols.

The extracellular matrix that supports organoid growth can also contribute to variable results. Many researchers use undefined mouse tumor-derived extracellular matrix with inherently high lot-to-lot variability. To develop standardized protocols and consistent results, the matrix ideally should be defined or at least screened to ensure compatibility with the required cell culture conditions.

The physical characteristics of organoids make analysis challenging. The organoid shape—from a simple sphere to more complex structures—affects analysis. At times, the same kind of organoid can vary in shape. These characteristics can make quantitative characterization difficult, especially since an organoid is thick enough that a typical compound microscope cannot image the entire structure. As a result, scientists often consider sectioning an organoid for analysis or using a more complicated form of deep-tissue imaging, which is still unlikely to provide a complete picture of an organoid's internal structure. Nonetheless, methods of multi-photon microscopy, such as two- and three-photon imaging, let scientists explore more deeply into organoids and view the entire structure in some cases.

Some of the challenges in organoid research could be addressed through collaborations and standardization of protocols across labs. Even standardizing the terminology used in the field would help. For instance, some publications still use the word "organoid" to describe what are commonly understood to be spheroids, or tissue-specific terms (e.g., enteroids or bronchosphere) that are not always used consistently.

Developing greater consistency in organoid cultures is key. The more that scientists develop and use consistent protocols and materials that can be more completely defined, the more data can be reproduced, compared, and the results applied in healthcare applications.

WHAT'S NEXT

Future benefits from organoids will arise not only from where they are applied, but how they are used. One example is monolayers. Organoids can be broken up and used to seed cultures made of a single layer, providing the cellular heterogeneity of an organoid with the convenience of 2D culture. van der Hee (2018) found that a monolayer made from organoids provided the ability to measure nutrient transport, barrier function, and interactions with gut bacteria.

Other structural adaptations include organoids being incorporated in organs-on-a-chip, which are devices that include a system of small channels that are used to provide nutrients to the cells inside. Boston-based Emulate notes that such systems can be used to study biology, improve human health, and develop new personal health applications. This company already offers lung, liver, and intestine chips. In the Netherlands, MIMETAS developed its OrganoPlate™, which the company describes as "a microfluidic 3D cell culture plate, supporting up to 96 tissue models on a single plate." MIMETAS has used their microfluidic system to generate models including perfused-gut epithelium tubules, various human neuronal and human liver models, and a model of the human kidney. By using cells grown in organoid cultures to populate the organ-on-a-chip system, researchers add a level of biological relevance and complexity not achievable with either system alone.

One of the great steps forward for organoids will be recognizing the true utility and limitations of the system, especially in clinical applications. For example, Berkers *et al.* (2019) created organoids from rectal tissue taken from patients with cystic fibrosis (CF). A compound called forskolin causes swelling in organoids created from healthy rectal tissue, but not from CF patients. Drug-driven improvements in CFTR activity triggered forskolin-induced swelling (FIS) in the CF-derived organoids; the same effect is produced in patients. Berkers *et al.* concluded: "*In vitro* drug efficacy measurements by FIS in rectal organoids of individuals with CF correlate

with the most important *in vivo* response indicators of CFTR modulators."
Despite this work and others working separately to accomplish similar
goals, more collaboration could be instrumental to see near-term impact on
patient care.

Another potential clinical application is cellular therapy via the func-
tional organoid transplantation. Cortez *et al.* (2018) transplanted human
intestinal organoids into the mesentery of immunosuppressed mice, after
which the organoids survived and grew. Various studies are already under-
way to explore the possibility of translating results generated from proof-
of-concept studies to clinical practice (Takebe *et al.*, 2018).

The work in organoids will continue to expand based on new and more
consistent methodologies. In addition, combining the results from other
technologies with those from organoids will also provide finer-grained
understanding of biological systems and open new pathways to disease
treatments.

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Surveys the history, methods, and applications of 3D cell culture.

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