Applications of human brain organoids to clinical problems

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Abstract

Brain organoids are an exciting new technology with the potential to significantly change how diseases of the brain are understood and treated. These three-dimensional neural tissues are derived from the self-organization of pluripotent stem cells, and they recapitulate the developmental process of the human brain, including progenitor zones and rudimentary cortical layers. Brain organoids have been valuable in investigating different aspects of developmental neurobiology and comparative biology. Several characteristics of organoids also make them attractive as models of brain disorders. Data generated from human organoids are more generalizable to patients because of the match in species background. Personalized organoids also can be generated from patient-derived induced pluripotent stem cells. Furthermore, the three-dimensionality of brain organoids supports cellular, mechanical, and topographical cues that are lacking in planar systems. In this review, we discuss the translational potential of brain organoids, using the examples of Zika virus, autism-spectrum disorder, and glioblastoma multiforme to consider how they could contribute to disease modeling, personalized medicine, and testing of therapeutics. We then discuss areas of improvement in organoid technology that will enhance the translational potential of brain organoids, as well as the possibility of their use as substrates for repairing cerebral circuitry after injury.

Introduction

The brain is an extraordinarily complex organ from which the entirety of human experience emanates. Understanding the intricacies of the human brain is thus one of the most fascinating endeavors in science. Equally compelling is the task of illuminating how failures in brain function manifest as neurological, cognitive, and psychiatric disorders. Two obstacles traditionally have stood in the way of these twin undertakings: the sheer complexity of the brain and difficulties in accessing human neural tissue. As such, the bulk of neuroscience research has resorted to the use of model systems, including cultures of dissociated neurons and animals. While these approaches have defined some of the fundamental principles of neurobiology and neurophysiology, they possess inherent shortcomings. Neuronal cultures lack the architecture of native brain tissue, which limits the investigation of interactions among different cell types. Species differences between humans and other animals dictate that information generated in the latter cannot necessarily be generalized to the former. Brain organoids are a relatively new technology that address, at least in part, these concerns and thus have the potential to transform how the brain and its associated diseases are studied.

In the most basic terms, organoids are simplified versions of organs that are grown *in vitro* (Lancaster and Knoblich, 2014b). Organoid technology has evolved tremendously from its earliest stages, in which dissociated embryonic chick cells were aggregated to form structures with a great deal of similarity to the organ of origin (Weiss and Taylor, 1960; Moscona, 1961). The current instantiation of organoids involves the generation of three-dimensional (3D) tissues from

pluripotent stem cells. Multiple cell types within the organoid are arranged in a manner that substantially resembles the emulated organ. This architecture arises from the principle of self-organization, an essential driver of normal development that encompasses the modes of self-assembly, self-patterning, and self-driven morphogenesis (Sasai, 2013).

The current era of human brain organoids began five years ago with the description of whole-brain organoids (commonly called "mini-brains" in the lay press)(Lancaster et al., 2013) and the generation of rudimentary neocortex with a multilayered structure and appropriate axial polarity (Kadoshima et al., 2013). These advances sparked intense interest and excitement for a variety of reasons. The faithful recapitulation of brain-specific structure in a laboratory-grown, 3D tissue hinted that brain organoids could be used to explore human neurodevelopment in previously unattainable ways. Moreover, as described in the initial study on whole-brain organoids (Lancaster et al., 2013), these entities potentially could serve as more relevant platforms for examining the mechanisms underlying disorders of the human nervous system. Several excellent reviews have summarized the issues and literature relevant to these topics (Lancaster and Knoblich, 2014b; Kelava and Lancaster, 2016; Kretzschmar and Clevers, 2016; Di Lullo and Kriegstein, 2017). In this review, we focus on translational aspects of brain organoids that are on the horizon, especially as they pertain to personalized models of pathogenesis, screening for therapeutics, and substrates for brain repair. We also discuss the major areas that require progress before the potential of cerebral organoids can be fully unlocked in a clinical context.

History and current state of brain organoids

Brain organoids represent the culmination of progress in several areas of biology (Lancaster and Knoblich, 2014b; Kelava and Lancaster, 2016). Building upon the chicken embryo studies described above (Weiss and Taylor, 1960; Moscona, 1961), the field of tissue engineering has refined methods for creating reproducible aggregates of cells (Ungrin et al., 2008; Dingle et al., 2015). Advances in stem cell biology have made available both fetus-derived embryonic stem (ES) cells (Thomson et al., 1998) and induced pluripotent stem (iPS) cells generated from adult somatic cells via cellular reprogramming (Takahashi et al., 2007). Lastly, rational application of knowledge gained from the study of neurodevelopment has yielded efficient protocols for producing neurons, such as the dual SMAD inhibition method (Gaspard et al., 2008; Chambers et al., 2009), that replicate the temporal sequence of neocorticogenesis (Shi et al., 2012; Espuny-Camacho et al., 2013).

These strands converged in the technique known as serum free culture of embryoid body-like aggregates with quick aggregation (SFEBq), which originally was developed as a variant of the embryoid body method for differentiating monolayer cultures of neurons (Watanabe et al., 2005). Maintaining the 3D configuration of SFEBq cultures and adjusting media components to support directed differentiation led to the self-formation of stratified structures such as the retinal cup (Eiraku et al., 2011; Nakano et al., 2012) and cortical epithelium (Eiraku et al., 2008; Kadoshima et al., 2013). Both spatial and temporal aspects of normal neocorticogenesis were maintained in the latter. Cortical tissues developed

identifiable zones that represented proper apico-basal polarity, including three progenitor zones (ventricular, subventricular, and intermediate) and three neuronal zones (subplate, cortical plate, and Cajal-Retzius cell). Features of rostral-caudal patterning also were present and could be manipulated using growth factors. Similar to adherent cultures, cortical plate neurons were born in the correct temporal sequence (lower layer before upper layer). A similar approach to SFEBq devoid of patterning growth factors and small molecule inhibitors resulted in brain organoids that modeled the developing brain in its entirety (Lancaster et al., 2013). These whole-brain organoids exhibited structural features reminiscent of the meninges, choroid plexus, and brain tissue. Markers of forebrain, midbrain, and hindbrain were present, and within the cortical domain, subregions consistent with different lobes of the cortex were identified.

Subsequent studies focused on improving the structural fidelity of cortex-specific organoids. A simplified approach for organoid generation that dispensed with the need for extracellular matrix supplementation (i.e., Matrigel) and media agitation yielded organoids with the well-defined segregation of superficial and deep cortical layers (Pasca et al., 2015). A miniaturized spinning bioreactor that decreased organoid-to-organoid variability produced organoids exhibiting a distinct layer of outer radial glial cells, an evolutionarily distinct hallmark of embryonic human cortex, and neurons belonging to all six cortical layers with rudimentary laminar separation (Qian et al., 2016; Qian et al., 2018). Formation of ellipsoid embryoid bodies around microfilament cores composed of poly(lactic-co-glycolic acid) and media supplementation with dissolved Matrigel reproducibly generated

elongated neuroepithelium with a radially organized cortical plate. Moreover, expansion of cortical folds in cortical organoids was induced by PTEN deletion, which increased the size of the progenitor pool (Li et al., 2017b). The genetic programs (Camp et al., 2015; Pasca et al., 2015; Qian et al., 2016) and epigenetic (Luo et al., 2016) and epitranscriptomic (Yoon et al., 2017a) signatures of these organoids generally matched those found in human fetal cortex up to the second trimester of development.

Although much of the initial emphasis of cerebral organoid studies centered on the development of glutamatergic cortical neurons, there has been growing interest in other cell types. The majority of cortical interneurons are born in the subcortical ganglionic eminences and populate the cortex via tangential migration (Chu and Anderson, 2015). Few interneurons are found in standard cortical organoids (Qian et al., 2016) because they are generated from protocols that promote dorsal cell fates and thus lack tissue resembling the ventral ganglionic eminences. To rectify this problem, ventralized organoids containing GABAergic neurons have been generated and fused to dorsalized glutamatergic organoids (Bagley et al., 2017; Birey et al., 2017; Xiang et al., 2017). These "assembloids" demonstrate the migration of interneurons into the dorsalized organoid and establishment of circuits with glutamatergic cells, mirroring normal developmental. With respect to glial populations, several studies have described the appearance of astrocytes (Pasca et al., 2015; Qian et al., 2016; Dezonne et al., 2017; Sloan et al., 2017) and possibly oligodendrocytes (Matsui et al., 2018) in cortical organoids at late time points. Organoids modeling the midbrain (Jo et al., 2016; Qian et al., 2016;

Monzel et al., 2017), hippocampus (Sakaguchi et al., 2015), pituitary gland (Ozone et al., 2016), hypothalamus (Qian et al., 2016), and cerebellum (Muguruma et al., 2015) also have been reported and are collectively referred to as region-specific brain organoids.

Insights into the neural activity of brain organoids have begun to emerge. Initial studies showed the presence of slow neuronal calcium waves and action potentials induced by current injections (Lancaster et al., 2013; Pasca et al., 2015; Qian et al., 2016). Spontaneous action potentials have been recorded with extracellular probes in eight-month-old but not four-month-old organoids, and the loss of this activity with the addition of neurotransmitter receptor antagonists suggests the presence of synaptic networks (Quadrato et al., 2017). Both glutamatergic and GABAergic spontaneous synaptic transmission can be detected in cortical organoids (Qian et al., 2016). Evoked action potential firing rates and excitatory synaptic inputs increase substantially in assembloids comprised of both glutamatergic and GABAergic neurons (Birey et al., 2017).

Translational applications of brain organoids

Organoids are an appealing model of the brain due to their human origin and because many of the cellular, mechanical, and topographical cues that arise from a 3D microenvironment are maintained (**Figure 1**). These advantages have been exploited to examine basic questions in developmental neurobiology, such as the genetic (Matsui et al., 2017; Mellios et al., 2018) and epitranscriptomic (Yoon et al., 2017a) control of cortical neurogenesis, and to investigate evolutionary differences

between human and non-human primate brains (Mora-Bermudez et al., 2016; Otani et al., 2016; Giandomenico and Lancaster, 2017). There has also been a surge in studies modeling human genetic diseases with organoids derived from patient-specific iPS cells and isogenic cell lines (Lancaster et al., 2013; Bershteyn et al., 2017; Iefremova et al., 2017; Ye et al., 2017; Allende et al., 2018). In addition to providing a unique platform for identifying the explanatory mechanisms underlying cerebral disorders, organoids may have considerable value in the realm of therapeutics as personalized test beds for drug screening and genomic manipulation. We will explore these translational themes using the examples of Zika virus-associated microcephaly, autism-spectrum disorder (ASD) as a representative of neurocognitive and neuropsychiatric disorders, and glioblastoma multiforme (GBM).

Zika virus-associated microcephaly

Zika virus (ZIKV) is a flavivirus first isolated from Ugandan rhesus monkeys in 1947 (Dick et al., 1952). It captured much public attention during an outbreak in Central and South American in 2015 after infections in pregnant women were linked to infants with abnormally small heads, a condition known as microcephaly (Mlakar et al., 2016; Ventura et al., 2016). Within a short period of time, ZIKV infection of the fetal brain was confirmed as the cause of microcephaly and other brain defects, and mechanisms of pathogenesis and potential drug therapies were identified. This rapid progress was facilitated by ZIKV studies in brain organoids, which synergized

with other approaches using monolayer cultures of neural progenitor cells, floating neurospheres, and human fetal tissue (Ming et al., 2016; Qian et al., 2017).

The recapitulation of fetal cerebral architecture in brain organoids has been particularly helpful in analyzing the effects of ZIKV infection on brain structure. An early study examining ZIKV pathogenesis in monolayer cultures suggested that human neural progenitors are more vulnerable to ZIKV infection than pluripotent stem cells or differentiated neurons (Tang et al., 2016). Subsequently, multiple studies have demonstrated that ZIKV infection results in smaller organoid size in the context of decreased cortical plate thickness and disruption of laminar organization (Cugola et al., 2016; Garcez et al., 2016; Qian et al., 2016). These findings would not have been possible in monolayer cultures. ZIKV preferentially infects ventricular and outer radial glial cells in cerebral organoids, depleting these progenitor pools through a combination of increased apoptotic cell death and suppressed proliferation (Qian et al., 2016). Brain organoids have been valuable in shedding light on the mechanisms underlying these observations. Upregulation of the innate immune receptor Toll-like Receptor 3 (TLR3) has been identified as a potential mediator of impaired neurogenesis in ZIKV-infected organoids via transcriptomic analyses (Dang et al., 2016). Early switching from horizontal to vertical division planes in dividing progenitors also has been found in ZIKV-infected organoids. This observation is consistent with premature progenitor differentiation as a contributor to progenitor pool depletion and cortical mantle thinning (Gabriel et al., 2017). Expression of the ZIKA-NS2A protein leads to the destabilization of adherens junction complexes and disorganized radial glial scaffolding in both

embryonic mouse cortex and human cerebral organoids (Yoon et al., 2017b). This last study is an excellent illustration of the experimental flexibility of organoids, which allows genetic manipulations via techniques such as electroporation and viral transduction to be used to elucidate pathogenic mechanisms.

Thus far, organoids have played a complementary role in the search for viable treatment strategies for ZIKV infection. High-throughput drug screens to identify ZIKV therapies have relied primarily upon monolayer cultures of iPS-derived human neural progenitor cells (Xu et al., 2016; Zhou et al., 2017). These screens and other hypothesis-driven approaches have revealed a number of candidate agents, which have been tested in monolayer cultures, animal models, and brain organoids (Xu et al., 2016; Li et al., 2017a; Sacramento et al., 2017; Watanabe et al., 2017; Zhou et al., 2017). Drug treatment of ZIKV-infected organoids has demonstrated decreased apoptosis (Xu et al., 2016; Watanabe et al., 2017), reduced virus replication (Sacramento et al., 2017; Zhou et al., 2017), and protection from infection (Li et al., 2017a; Watanabe et al., 2017). The use of organoids to assess the efficacy of drugs on preserving the structural integrity of cortex has not yet been performed. This approach may have the most relevance to patients and thus could be especially important in validating drug-testing results from other models.

Cell surface receptors that mediate ZIKV entry into cells highlight another role for organoids in identifying targets for therapeutic intervention. The TAM receptor protein AXL was thought to be a strong candidate for this role in part because of its expression in many tissues that correlate with the path of viral transmission from mother to fetus (Ming et al., 2016). High expression of AXL also is

found in radial glial cells, astrocytes, endothelial cells, and microglia in human fetal brain tissue (Nowakowski et al., 2016). However, deletion of AXL using genome-editing techniques did not alter ZIKV entry into or its deleterious effects on human neural progenitors in monolayer cultures or brain organoids (Wells et al., 2016). Other studies have found that AXL mediates ZIKV entry into human glial (Meertens et al., 2017) and endothelial cells (Richard et al., 2017), suggesting an alternative role for this receptor in ZIKV pathogenesis. These findings emphasize the need to consider organoid studies in the context of other disease models.

Autism spectrum disorder

Autism spectrum disorders are a group of neurodevelopmental conditions characterized by deficits in social interactions, communication, and behavioral flexibility (Ilieva et al., 2018). The genetic basis of ASD is quite complex. Many candidate genes, each with multiple pathogenic variants, have been identified, and this molecular diversity is likely responsible for the heterogeneity seen in clinical manifestations (Geschwind, 2011). Explanatory models for ASD include impaired synaptic development (Zoghbi, 2003), abnormal neural connectivity secondary to compromised activity propagation and information processing (Belmonte et al., 2004; Courchesne and Pierce, 2005), and developmental disconnection specifically within the frontal and temporal regions (Geschwind and Levitt, 2007).

It stands to reason that human models are most suitable for studying ASD because of the involvement of the cerebral cortex, which is more highly developed in humans than other species, in ASD pathology (Parikshak et al., 2013). Moreover,

the hypothesized role of neural connectivity in ASD pathology is best investigated with networks comprised of discrete groups of neurons to detect problems with neural activity timing and multiple neuron types to faithfully simulate *in vivo* circuits. Brain organoids fulfill these criteria. It is also expected that organoids, by virtue of their cellular variety, cell-matrix interactions, and re-constitution of *in vivo* cytoarchitecture and niche-like environments, will better maintain the *in vivo* genetic and epigenetic landscape than monolayer cultures (Forsberg et al., 2018). Organoids derived from individuals with severe idiopathic ASD demonstrate accelerated cell-cycle dynamics and FOXG1-dependent overproduction of interneurons (Mariani et al., 2015). Patients in this study did not carry any genetic mutations with known associations with ASD, which underscores the potential of organoids to serve as personalized models of disease pathogenesis in highly polygenic disorders (Arlotta, 2018). The opposite approach of examining the contribution of a specific gene to ASD in organoids has also been tried. Isogenic iPS cell lines that are heterozygous for a *CHD8* knockout allele have been used to generate cerebral organoids for transcriptomic analysis. *DLX6-AS1* and *DLX1*, which are involved in interneuron differentiation, are among the top differentially expressed genes in *CHD8* knockout heterozygote compared to control organoids (Wang et al., 2017). Similar studies using patient-derived organoids and organoids overexpressing a disease gene have been performed in schizophrenia (Ye et al., 2017). These studies are the vanguard of organoid-based investigations of neuropsychiatric and neurocognitive disorders. Additional progress in this direction

will need to resolve several questions, including the optimal outcome metrics that are most relevant to clinical symptoms (Ilieva et al., 2018).

Brain tumors

Glioblastoma multiforme (GBM) is the most common primary brain malignancy in adults (Ostrom et al., 2017), and it remains one of the most difficult tumors to treat with a median life expectancy of only 15 months (Stupp et al., 2005; Stupp et al., 2017). A significant part of the challenge in treating this disease is its significant intratumoral heterogeneity (Sottoriva et al., 2013; Parker et al., 2015) and highly infiltrative nature in the brain. Organoid technology may be suitable for modeling both these features with implications for understanding the pathophysiology of GBM and developing patient-specific therapies.

Like many other tumors (Ebben et al., 2010), GBM is thought to arise from mutated stem cells (Sanai et al., 2005). Neurospheres consisting of these putative glioma stem cells have been isolated from patient tumor samples (Ignatova et al., 2002; Galli et al., 2004), and they can differentiate into multiple cell types along neuronal, astroglial, and oligodendroglial lineages (Gunther et al., 2008). However, tumor formation typically occurs only after xenograft transplantation (Gunther et al., 2008; Schulte et al., 2011). Applying the principles of organoid formation to glioma neurospheres and patient tissue in general could result in glioma organoids that reflect the structure, heterogeneity, and perhaps microenvironment of GBM *in vivo*. In essence, glioma organoids would be 3D human representations of tumors that are free of the constraints of an animal host. An initial report of organoids

derived from patient GBM and cerebral metastasis tissues has demonstrated the feasibility of this approach (Hubert et al., 2016). This study showed phenotypic heterogeneity among stem cells in the glioma organoids, as well as hypoxic gradients that modulated mitotic activity in stem cell niches. Glioma organoids also have been generated by introducing the HRas^{G12V} oncogene into whole-brain organoids via CRISPR/Cas9-mediated homologous recombination in the TP53 locus (Ogawa et al., 2018). Transformed cells rapidly proliferate and invade the "healthy" organoid, overtaking the bulk of the organoid after 16 weeks. The molecular profile of these tumor cells is most consistent with the highly aggressive mesenchymal subtype of GBM. Similarly, deletion of DNA repair genes in colonic organoids via CRISPR/Cas9 techniques has been performed to study the accumulation of mutations that drive oncogenesis (Drost et al., 2017). Further investigations along these lines could further elucidate the etiology and evolution of intratumoral heterogeneity, factors influencing the tumor cell-microenvironment interaction, and other pathophysiological mechanisms in GBM.

Glioma organoids also could contribute to the development of personalized therapeutics for GBM patients, which is necessitated by the high degree of genetic variability in this tumor. The effectiveness of different treatment regimens could be trialed in glioma organoids, allowing therapeutic effects on the stem cell versus non-stem cell compartment of the tumor to be evaluated before a course of action is chosen for a patient. This type of organoid biobank approach for treatment screening has been applied to be breast (Sachs et al., 2018), gastrointestinal (Vlachogiannis et al., 2018), and bladder cancers (Lee et al., 2018).

Organoid fusion may be a novel strategy for investigating mechanisms involved in the spread of GBM in the brain. Mimicking the fusion of dorsalized and ventralized organoids to study interneuron migration, glioma organoids could be fused to normal brain organoids to assess for tumor cell infiltration. Along these lines, GBM neurospheres merged with cerebral organoids have demonstrated the ability to infiltrate the organoid (da Silva et al., 2018; Ogawa et al., 2018). This strategy could be used to assess the temporal dynamics and cellular mechanisms of infiltration. In particular, genetic and epigenetic analyses of infiltrative versus non-infiltrative tumor cells in fused organoids could help define molecular signatures of infiltration and suitable targets for therapeutic intervention. Such therapies could then be tested by assaying tumor cell infiltration in fused organoids.

Areas for improvement

The above examples demonstrate the ample advantages of using organoids to model brain diseases. First and foremost, brain organoids are human systems that better match the genetic background and structural characteristics of the human brain than animal models. They are also much more accessible and available in greater quantities than human brain tissue. Lastly, organoids are 3D structures that recapitulate many aspects of the *in vivo* environment of brain tissue, including its cytoarchitecture, cellular variability, cell-cell and cell-matrix interactions, and various cellular niches. Such features are missing in two-dimensional monolayer cultures. Fully exploiting the benefits of brain organoids as models will require disease-specific fine-tuning. There also exist areas of improvement that cut across

all organoid applications to clinical problems, including the logistics of organoid generation, fidelity of brain emulation, and characterization of neural circuit structure and activity.

Logistics of organoid generation

Variability in brain organoid generation imposes constraints on their use for translational purposes. Batch-to-batch variability can be significant, especially with whole-brain organoids (Lancaster and Knoblich, 2014a). Single-cell sequencing experiments show that organoids grown in the same bioreactor exhibit a higher degree of similarity than organoids obtained from different bioreactors and that particular cell populations are only present in certain bioreactor batches (Quadrato et al., 2017). This degree of variability necessitates the inclusion of larger numbers of organoids in studies to mitigate outlier effects. Various techniques have been employed to decrease this cellular variability, including miniaturized spinning bioreactors that may produce a more consistent environment for organoid growth (Qian et al., 2016) and increasing the surface area of embryoid bodies relative to their volume to improve the efficiency of neural induction (Lancaster et al., 2017). An additional source of variability is the number of cortical zones present in each organoid. The factors governing the formation of cortical zones are not yet clear but may include the initial attributes of embryoid bodies (e.g., number of cells, size, and shape), biomechanical variables (Varner et al., 2015), and developmental cues. Simple dissection, as has been done with retinal organoids (Volkner et al., 2016), could be a straightforward way of isolating individual cortical zones. Further

application of principles drawn from developmental neurobiology and bioengineering, as well as quality control practices, will greatly improve the reproducibility of organoid generation and efficiency of organoid-based disease models.

Another limitation of cerebral organoids for modeling purposes is the long length of time required for growth and maturation. Similar concerns are relevant to any human stem cell-based platform. Several months are necessary to obtain organoids with molecular signatures similar to second-trimester fetuses (Pasca et al., 2015; Qian et al., 2016). Ostensibly, additional lengths of time would be needed to mature organoids to the third trimester and post-natal stage. While current brain organoids are adequate for modeling neurodevelopmental disorders and fetal infections, there are legitimate concerns about their applicability to cerebral disorders with later onsets, such as schizophrenia, which typically manifests in adolescence (Soliman et al., 2017), and neurodegenerative diseases (Vera and Studer, 2015). Methods for accelerating maturation are being sought and include over-expression of genes implicated in premature aging disorders (Miller et al., 2013; Zhang et al., 2015) and PTEN deletion (personal communication, S. Anderson).

Finally, high-throughput screening methods based on organoids are just beginning to emerge. The previously described miniaturized spinning bioreactor has helped scale up the generation of cerebral organoids (Qian et al., 2016). However, most analyses still require multiple, time-consuming steps, including those associated with immunohistochemistry and microscopy. Newer imaging

techniques such as tissue clearing (Chung et al., 2013; Renier et al., 2014) and imaging mass cytometry (Giesen et al., 2014) have the potential to streamline image analysis, but there is a need to develop the methods to analyze multiple samples simultaneously. To this end, radiofrequency identification chips have been embedded in human liver organoids, enabling the identification of disease phenotypes (Kimura et al., 2018). Combining organoids with other tissue matrix arrays (Beachley et al., 2015) or organ-on-a chip technologies (Bhatia and Ingber, 2014) may be alternative solutions to this multiplexing issue.

Augmenting cerebral organoid complexity

Brain organoids in their current iterations already model a great deal of the brain's complexity, but there are important features that are still missing. Most recent efforts in the field have focused on brain region-specific organoids because it is easier to control their development and structure than whole-brain organoids. This approach is limited in examining interactions between different areas of the brain. Fusion of different brain region-specific organoids provides a means of studying these systems-level interactions. For example, assembloids of cortical and thalamic organoids could be used to model human cortico-thalamic circuitry, which underlie all cortical functions. One downside of this strategy and of cerebral organoids in general is the lack of axon tracts, which serve as the substrate for cerebral connectivity. Novel bioengineering techniques, including axon stretch growth (Pfister et al., 2004) and hydrogel micro-conduits (Cullen et al., 2012; Struzyna et al., 2015), could incorporate axon bundles into organoid models, which

would enable the study of neural activity propagation between network nodes (Chen et al., 2017).

Another issue with current brain organoids is the lack of endothelial cells, microglia, and other immune cells, which are valuable for disease modeling. The available protocols for generating brain organoids direct cellular differentiation exclusively along ectodermal pathways. As such, they do not contain any cells that originate from the mesoderm. The blood-brain barrier is imperative for maintaining cerebral homeostasis, and its disruption is implicated in a number of diseases (Zhao et al., 2015b). Interactions between neural stem cells and the cerebral vasculature, the so-called perivascular niche, may be involved in normal progenitor proliferation and differentiation (Shen et al., 2008; Tavazoie et al., 2008; Codega et al., 2014). Microglia are the resident immune cells of the brain, and they have been implicated in both healthy brain function and pathological neuroinflammation (Blank and Prinz, 2013; Colonna and Butovsky, 2017). Infiltrating monocytes and T cells contribute to the complex microenvironment of GBM (Domingues et al., 2016; Gabrusiewicz et al., 2016), and understanding their role in the immunosuppressed status of this tumor is crucial for optimizing immunotherapies such as CAR-T cells (O'Rourke et al., 2017). Co-culturing techniques could be adequate to introduce these missing cell types into cerebral organoids (Pham et al., 2018), especially in the case of immune cells that are naturally infiltrative. Other methods may need to be developed in order to fully integrate vascular networks and immunological compartments into brain organoids.

Increasing the size of cerebral organoids and maintaining their health during prolonged periods of growth are likely pre-requisites for promoting their maturation beyond the 2nd trimester of human fetal development. As discussed above, developing more mature organoids is particularly important for more accurately modeling cerebral diseases affecting later stages of the human life cycle. The major obstacle to achieving this goal has been inadequate nutrient, gas, and waste exchange in *in vitro* culture, which leads to a necrotic organoid core and suboptimal cell health beyond the superficial layer of the organoid. Media agitation (Lancaster et al., 2013; Qian et al., 2016) and hyperoxic culture conditions (Kadoshima et al., 2013) have maximized the efficiency of diffusion and mass transport. Further organoid growth and development will need the equivalent of a circulatory system. Micro-fluidics and other bioengineering techniques have improved the ability to perfuse 3D engineered tissues (Miller et al., 2012; Hasan et al., 2014). Another approach is to use host animals as "bioreactors" to support the growth of cerebral organoids. It was shown recently that human brain organoids survive and integrate after transplantation into mouse cortex (Mansour et al., 2018). Grafted organoids are extensively vascularized by the host two weeks after transplantation and contain significantly fewer apoptotic cells than age-matched organoids *in vitro*. Furthermore, progressive differentiation of neurons, gliogenesis, and host microglia infiltration is observed within the transplanted organoid.

Organoid transplantation could be an end unto itself as a strategy for brain repair. The vast majority of the current approaches for rebuilding cerebral circuitry results in grafted neurons that are arranged in a fairly haphazard manner (Guo et al.,

2014; Michelsen et al., 2015; Somaa et al., 2017; Tornero et al., 2017). Especially in highly structured areas of the brain such as the cerebral cortex and hippocampus, a reasonable expectation is that grafts with a greater degree of brain-specific architecture would more effectively restore cerebral function (Chen et al., 2016). Brain organoids are one of the few available substrates that recapitulate cerebral architecture and thus could be the starting point for investigating a new approach to brain repair. Many of the improvements discussed in this section may prove beneficial for the usage of brain organoids as repair substrates as well.

Defining neural activity and networks in brain organoids

A better understanding of how neural activity develops in brain organoids will enhance their modeling of neuropsychiatric and neurocognitive disorders (Di Lullo and Kriegstein, 2017). Neural activity arising from and between neural ensembles is the closest proxy to behavior and clinical function available in the laboratory. Thus, it is likely to be one of the best outcome metrics available for organoid-based models.

The current picture of neural activity evolution in brain organoids is incomplete. Spontaneous action potentials measured with extracellular techniques have been observed in eight-month-old but not four-month-old organoids (Quadrato et al., 2017). A more precise timeline for the appearance of these spontaneous action potentials remains to be determined, and it will be interesting to compare this timeline to the evolution of spiking activity in human fetal brain tissue (Moore et al., 2011). Moreover, spontaneous calcium activity and evoked action

potentials have been documented in cerebral organoids well before eight months (Pasca et al., 2015; Qian et al., 2016). The relationship between this nascent activity and spontaneous action potentials is not known. Perhaps most importantly, very little is known about the development of local neural circuits and larger neuronal networks in brain organoids. Planar cultures of iPS cell-derived human cortical neurons may provide some guidance in this area. These cultures transition from asynchronous activity to synchronized oscillatory networks to structured asynchronous activity with recurring activity motifs over 100 days (Kirwan et al., 2015). It is clear that the presence of interneurons accelerate the functional maturity of neurons in brain organoids (Birey et al., 2017), but other factors pertinent to this maturation process, including the contribution of astrocytes and other glial cells, have not yet been examined in detail. Making progress on these various topics will require collaborative work among developmental neurobiologists and circuit-/systems-level neuroscientists.

Clinical and ethical considerations

In addition to technical improvements in generating and understanding brain organoids, there are issues associated with the clinical application of these entities that require careful consideration. The long length of time needed to generate patient-specific iPS cells and brain organoids is a major impediment to the practical use of the latter for both brain modeling and circuit repair. Methods for accelerating the growth of brain organoids have been discussed above. An alternative could be the creation of organoid banks for specific patient genetic

profiles, immunological phenotypes, and disease types. The complex microenvironment of brain organoids may predispose them to genetic, epigenetic, and phenotypic drift over time, which could impact their ability to effectively model cerebral disorders. Quality-control protocols and thorough validation of organoid-derived data for patient use could help manage such problems. Finally, there are potential risks to using brain organoids for brain repair, which overlap with concerns associated with stem cell-based therapies in general. Even patient-derived iPS cells, and hence brain organoids, could provoke an immune reaction (Zhao et al., 2011; Zhao et al., 2015a). Oncogenic transformation of transplanted progenitor cells remains a worry, and over-exuberant organoid growth that is not neoplastic also could cause neurological problems. Mechanisms to limit organoid growth or induce apoptosis of transplanted cells may be needed to safeguard against these eventualities. Transplanted organoids could trigger unwanted functional outcomes, including seizure activity. Neuromodulatory interventions may be necessary to facilitate the proper integration of organoid neurons and prevent maladaptive plasticity.

The generation of human brain organoids has spurred ethical debates on a range of topics, which prompted two workshops supported by the National Institutes of Health in May 2017 and March 2018 (Farahany et al., 2018). There are three general areas of discussion. The first pertains to the moral status and well being of the organoids themselves. Because of their human origin and brain-like architecture, brain organoids theoretically have the potential to attain more advanced brain states, including sensory perception (e.g., pain) and perhaps

consciousness. The likelihood that current brain organoids are conscious is miniscule given their small size and primitive nature. However, as their size and complexity increase, pertinent questions include defining sensitive methods for assessing possible sentience (Lavazza and Massimini, 2018) and determining the need to protect these more advanced organoids in ways similar to laboratory animals. Second, transplantation of human brain organoids into animal hosts will create a spectrum of human-animal chimeras. While chimeras have become more commonplace in biomedical research, brain chimeras may require special consideration. Is it scientifically feasible for brain chimeras to become enhanced or more human-like? If so, are there certain levels of enhancement that would be considered acceptable, and would these enhanced chimeras deserve additional research protections? Lastly, there are issues related to consent and ownership. When subjects donate their cells for the generation of iPS lines, should they be told explicitly that brain organoids will be generated from these lines? Can they request specifically that brain organoids not be generated? As brain organoids become more complex, the question of who controls the fate of these entities (e.g., the researcher, source subject, or perhaps state) also may become more contentious. Further discussions of these issues should include all relevant stakeholders and be based upon appropriately defined scientific frameworks and ethical principles.

Conclusion

Brain organoids have inspired such intense excitement over the past five years because they are the only *in vitro* platform that recreates the 3D architecture

of the human brain and recapitulates the process of human neurodevelopment. Beyond the study of normal brain development, organoids provide a unique opportunity to model human brain disorders and develop therapies for these conditions. The examples of Zika virus, autism-spectrum disorders, and glioblastoma multiforme underscore three translational themes. First, due to their human and 3D nature, brain organoids enable the investigation of aspects of disease pathogenesis that are difficult or impossible to examine using other models. Second, the possibility of generating patient-specific organoids means that personalized models of disease can be created, which may be especially helpful in understanding the pathogenesis of polygenic disorders. Third, brain organoids may provide unique outcome measures that can be used to screen the efficacy of drugs and other therapeutic interventions in a personalized manner. Separate from disease modeling, brain organoids are a potentially transformative substrate for rebuilding cerebral circuitry after injury. All of these translational indications would benefit from continued improvements in the reproducibility, maturity, and biological complexity of organoid technology. While progress is being made on these fronts, it will be important to keep in mind potential ethical quandaries associated with cerebral organoid growth and use. Resolving these biological, engineering, clinical, and ethical issues will help realize the full potential of brain organoids in their application to clinical problems.

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Figure 1. Benefits and disadvantages of different model systems for human brain disorders. Conventional models of brain disease include monolayer cultures of stems cells, neural progenitors, or neurons; animal models; and human neural tissue. Brain organoids are an intriguing new technology that addresses many of the shortcomings of these other platforms. They may allow disease pathogenesis and therapeutic testing to be explored in previously unachievable ways. 3D: three-dimensional, iPS cells: induced pluripotent stem cells.

Planar cultures	Animal models	Human tissue	Brain organoids
Pros: - Derivable from patient iPS cells - Readily available - Easy to manipulate - High throughout **Cons:** - Lacks brain cytoarchitecture - Lacks 3D microenvironment - Missing many cell types	**Pros:** - Intact brain structure - 3D microenvironment - Behavioral testing **Cons:** - May not generalize to patients - Low throughput	**Pros:** - Human origin - Intact brain structure - 3D microenvironment **Cons:** - Limited availability - Genetic manipulations difficult - Low throughout	**Pros:** - Human origin - Derivable from patient iPS cells - Brain-like cytoarchitecture - 3D microenvironment **Cons:** - Limited maturity & size - Missing certain cell types - Neural activity not well defined