Dual Perspectives

Dual Perspectives Companion Paper: Integrating CRISPR Engineering and hiPSC-Derived 2D Disease Modeling Systems,
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Studying Human Neurodevelopment and Diseases Using 3D Brain Organoids

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In vitro differentiation of pluripotent stem cells provides a systematic platform to study development and disease. Recent advances in brain organoid technology have created new opportunities to investigate the formation and function of the human brain, under physiological and pathological conditions. Brain organoids can be generated to model the cellular and structural development of the human brain, and allow the investigation of the intricate interactions between resident neural and glial cell types. Combined with new advances in gene editing, imaging, and genomic analysis, brain organoid technology can be applied to address questions pertinent to human brain development, disease, and evolution. However, the current iterations of brain organoids also have limitations in faithfully recapitulating the *in vivo* processes. In this perspective, we evaluate the recent progress in brain organoid technology, and discuss the experimental considerations for its utilization.

Key words: Brain organoid; pluripotent stem cells; CRISPR; neurological disorders; evolution

Introduction

The human brain is one of the most complex organs in the human body. Much of our current knowledge about the molecular, cellular, and anatomical mechanisms of human brain development was gained by studying model organisms, which revealed that many aspects of brain development are evolutionarily conserved. However, the human brain is also substantially different from those of other species, including rodents and nonhuman primates, in size, shape, cellular composition, and ultimately, the capacity for higher cognitive function. Many of these differences are deeply rooted during fetal development and are already apparent at birth. At the structural level, the human brain, in particular the cerebral neocortex, is greatly expanded in size, driven by an increase in the number of neurons and glia as well as the overall cortical area (Bystron et al., 2008; Sun and Hevner, 2014). This expansion is accompanied by the emergence of extensive cortical folding.

At the cellular level, neural stem and progenitor cells undergo highly controlled proliferation and differentiation processes to generate the vast numbers of neurons and glia that make up the postnatal brain. It has been observed that human neural stem and progenitor cells have enhanced proliferative capacity, and are intrinsically more diverse than their counterparts in other species, such as the mouse and the rat (Rakic, 1974; Otani et al., 2016). Dysfunctions of these stem and progenitor cells have been linked to structural malformations of the brain (Walsh, 1999). Disruptions of their differentiated neuronal and glial progenies are associated with neurodevelopmental disorders ranging in severity (Hu et al., 2014). Many of these disorders have been proven difficult to study in animal models. Therefore, direct analysis of human neural cells and tissues is required to enhance our understanding of both conserved and nonconserved mechanisms governing normal and pathological human neurodevelopment.

The advent of human pluripotent stem cell (hPSC) technologies, including that of embryonic stem cells and induced pluripotent stem cells (iPSCs), opened a new avenue to study human neural development and disease *in vitro*. Directed differentiation of hPSCs into the neural lineage can now be efficiently performed in adherent 2D cultures (Zhang et al., 2001) (also see accompanying Dual Perspectives article by Rehbach et al.). It has long been observed that neural differentiation can occur efficiently in embryoid bodies, aggregates of PSCs cultured in suspension (Itskovitz-Eldor et al., 2000). Variants of the embryoid body-based differentiation methods were shown to yield complex neu-

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roectodermal structures resembling the cerebral cortex and the optic cup, demonstrating the feasibility of recapitulating organogenesis *in vitro* using 3D differentiation and highlighting the ability of hPSCs and their progenies to self-organize (Eiraku et al., 2008; Nakano et al., 2012). Building on these efforts, and integrating successful methods developed to generate 3D intestinal epithelium structures from mouse intestinal stem cells (Sato and Clevers, 2013), Lancaster et al. (2013) developed protocols to generate cerebral organoids from hPSCs. Relying on the intrinsic propensity for hPSCs to differentiate toward the neural lineage, these 3D neural tissues harbor broad regional identity, such as the forebrain, midbrain, and hindbrain, and display complex structural organization reminiscent of the cytoarchitecture of the fetal human brain, including the formation of polarized cortical tissue and fluid-filled ventricles (Lancaster et al., 2013; Lancaster and Knoblich, 2014). Within these cerebral organoids, neuroepithelia are induced from PSCs, which give rise to neural stem and progenitor cells and undergo differentiation into diverse subtypes of neurons and glia. Single-cell RNA sequencing analysis revealed substantial similarities in gene expression profiles between cerebral organoid and fetal human cerebral cortex (Camp et al., 2015). It is worth noticing that non-neural lineages can also be derived in these self-patterned cerebral organoids due to unguided differentiation and insufficient inhibition of mesoderm and endoderm lineages (Camp et al., 2015; Quadrato et al., 2017; Ormel et al., 2018). Further incorporation of patterning cues allowed the generation of organoids with exclusive neural identity and organoids with specific brain-region or cell-type identities (Kadoshima et al., 2013; Mariani et al., 2015; Muguruma et al., 2015; Pasca et al., 2015; Sakaguchi et al., 2015; Qian et al., 2016).

The development of brain organoid technology expands the already diverse possibilities of using PSCs to study human neurobiology. Compared with adherent 2D neural differentiation, the process of brain organoid generation preserves the spatial architecture that forms as the primitive neural tissues self-organize, and allows the investigation of early human brain formation, a process difficult to observe *in vivo* and that diverges significantly between human and experimental organisms. Long-term culture of brain organoids encourages development of stable cell–cell interaction at physiological proximity and density, which is particularly relevant for the formation of meaningful connections and interactions between neuronal cells and among neurons and glia. In the short span of a few years, brain organoids have been used to model human brain development, neurological disorders, and evolution. The development of organoid technology also coincided with rapid advances in genome editing, single-cell genomics, tissue clearing, and imaging techniques, all of which are adaptable and well suited for application in brain organoids. Here, we explore the various use of brain organoid technologies and discuss experimental considerations for incorporating this tool into a discovery process.

Applications and limitations of brain organoid models

Studying human brain development

In vitro differentiation of hPSCs has revolutionized our ability to study early development and cell fate transition. In the absence of induction toward other lineages, PSCs adopt a default path into the neural fate (Muñoz-Sanjuán and Brivanlou, 2002). In 3D suspension culture, as in 2D adherent cultures, hPSCs can spontaneously differentiate into cell types characteristic of the primitive neuroepithelium (Eiraku et al., 2008). While self-assembly and self-patterning emerge in both types of cultures, continuous culturing of organoids in suspension without dissociation has the

Figure 1. Cross section of a day 83 human forebrain organoid, showing MAP2⁺ neurons (green) and SOX2⁺ neural precursors (red), surrounding a ventricle-like structure. DAPI (blue). Scale bar, 20 μ m.

advantage of allowing uninterrupted self-organization into *in vitro* organogenesis (Fig. 1). This provides an opportunity to closely study this early developmental process, in particular the features that diverge between human and experimental organisms, such as the mouse.

It has been shown that, while brain organoids from both murine and human PSCs develop stratified structures resembling ventricular zone, subventricular zone, intermediate zone, and cortical plate, only human brain organoids develop outer subventricular zone-like structures (Eiraku et al., 2008; Kadoshima et al., 2013; Lancaster et al., 2013). This recapitulates the presence of the outer subventricular zone *in vivo* in the human, but not the mouse cortex. Within the germinal zones of human brain organoids, proliferative precursors resembling ventricular and outer radial glial cells are generated, in a temporal and spatial pattern consistent with *in vivo* human cortical development (Kadoshima et al., 2013; Lancaster et al., 2013). Of note, proliferation of precursors in the ventricular zone and subventricular zone persists for a prolonged period in the human brain organoids, compared with their mouse counterparts (Lancaster et al., 2013; Y. Li et al., 2017). As radial glial cells differentiate into intermediate progenitors, and later into neurons and glia, the cell types generated recapitulate the transcriptomic and epigenomic signatures of the human fetal brain (Camp et al., 2015; Luo et al., 2016; Quadrato et al., 2017; Amiri et al., 2018; Pollen et al., 2019). Differentiating cortical neurons display inside-out migratory patterns consistent with *in vivo* development, and establish rudimentary cortical layering according to their time of birth (Kadoshima et al., 2013; Lancaster et al., 2013).

To model long distance neuronal migration between distinct brain regions, brain organoids can be prepatterned to develop region-specific identity, after which different organoids can be cocultured to allow physical contact and fusion. Using this approach, directional migration of inhibitory neurons from ventral cortical organoids into dorsal cortical organoids has been observed (Bagley et al., 2017; Birey et al., 2017; Xiang et al., 2017;

Sloan et al., 2018). Migration of human interneurons in brain organoids recapitulates the saltatory pattern seen during fetal human cortical development and is distinct from that of mouse interneurons (Birey et al., 2017).

As brain organoids mature with prolonged culture, functional neuronal connections are formed, allowing the observation of neuronal activity on the levels of cells and network. Spontaneous and evoked neural activity can be measured using calcium imaging (Lancaster et al., 2013; Paşca et al., 2015; Qian et al., 2016), current/voltage clamp (Paşca et al., 2015; Qian et al., 2016), and multielectrodes (Kawada et al., 2017; Quadrato et al., 2017; Giandomenico et al., 2019). Maturation of astrocytes (Paşca et al., 2015; Sloan et al., 2017) and oligodendrocytes (Madhavan et al., 2018; Marton et al., 2019) can also be seen, allowing the investigation of the development and function of these glial cell types.

Modeling human neurological disorders

An array of neural developmental and degenerative disorders has been successfully modeled using brain organoids. Because the course of brain organoid generation approximates the early steps of fetal brain formation, diseases that impact this developmental process were among the first to be successfully modeled. For example, several early studies modeled diseases that result in reduced neural structural formation, such as genetic forms of microcephaly (Lancaster et al., 2013; Jin et al., 2017; Omer Javed et al., 2018). After maternal exposure to Zika virus was linked to a range of fetal brain abnormalities, including microcephaly, lissencephaly, and cortical calcification, brain organoids were used to investigate the cause of the neuropathology and to identify potential therapeutic strategies (Garcez et al., 2016; Qian et al., 2016; Xu et al., 2016; Y. Li et al., 2017, 2019; Watanabe et al., 2017; Zhou et al., 2017). At the other end of the spectrum, enhanced and excessive growth of the fetal brain has been reported in human macrocephaly and polymicrogyria, including those caused by mutations in the AKT-mTOR signaling pathway (McBride et al., 2010; Lee et al., 2012; Poduri et al., 2012; Rivière et al., 2012). Genetically modified brain organoids with elevated AKT-mTOR signaling activities have been used to model these enhanced growth conditions (Y. Li et al., 2017). In addition, abnormal neural migration, which occurs in genetic forms of lissencephaly and periventricular heterotopia, caused by mutations in genes, such as *DCHS1*, *FAT4*, or *PLEKHG6*, has been modeled in brain organoids (Bersteyn et al., 2017; O'Neill et al., 2018; Klaus et al., 2019).

In addition to studying the mechanism underlying diseases involving well-defined brain malformation, organoids derived from hPSCs bearing causal mutations for neurodevelopmental and psychiatric diseases can be used to identify previously unrecognized abnormalities. For instance, altered neurogenesis and imbalance in neuronal and glial differentiation have been reported in organoid models of several neurodevelopmental disorders, including autism spectrum disorders (Mariani et al., 2015), Rett syndrome (Mellios et al., 2018), tuberous sclerosis (Blair et al., 2018), and *DISC1*-related schizophrenia (Ye et al., 2017; Srikanth et al., 2018).

Brain organoids have also proven advantageous in modeling neurodegenerative diseases and other neurological conditions. Because 3D culturing allows the formation of long-term cell–cell contacts at physiological density, it facilitates the retainment of pathological protein aggregates that are often the hallmark of neurodegenerative diseases. Increased accumulation of amyloid- β peptide and phosphorylated tau protein has been reported in organoid models of familial and sporadic forms of Alzheimer's disease (Raja et al., 2016; Lin et al., 2018), whereas increased levels of phosphorylated

α -synuclein have been reported in an organoid model of Parkinson's disease with mutation in the *LRRK2* gene (Kim et al., 2019). Accumulation of these protein aggregates has previously been difficult to detect and preserve using 2D adherent cultures. Organoids have also been used to model brain tumor initiation and progression. Introduction of known oncogenic mutations was shown to result in neoplastic cerebral organoids recapitulating features of glioblastoma and CNS primitive neuroectodermal tumors, enabling the study of the crucial first steps in the genetic transformation of normal neural stem cells into brain tumor initiating cells (Bian et al., 2018). In addition, transplantation of glioma stem cells from patient biopsy allowed the investigation of tumor invasion into host brain tissue, providing a novel human-based xenograft platform to assess disease mechanism and drug responses (Linkous et al., 2019).

Investigating the mechanisms of brain evolution

The development of PSC technology presents an unprecedented opportunity to perform interspecies comparative genomics studies. Our ability to derive neural cells and organoid tissues from these PSCs, combined with an ever-expanding toolkit for genome editing, allows functional interrogation of the underlying mechanisms that drive brain evolution. Comparison of neural cells derived from human, chimpanzee, and macaque PSCs demonstrated species-specific duration of proliferation and cumulative neuronal output (Otani et al., 2016). Of note, human neural stem and progenitor cells displayed longer periods of expansion in both 2D adherent cultures and 3D brain organoids than cells from other species (Otani et al., 2016). While the underlying mechanism for the species-specific cellular behaviors is likely multifaceted, recent studies indicate that human-specific gene duplication may be a contributing factor (Florio et al., 2015, 2018; Fiddes et al., 2018; Suzuki et al., 2018). Features of species-specific cortical development, such as the differential abundance of outer radial glial cells, could be detected in brain organoids derived from human and non-human primates (Pollen et al., 2019).

Interspecies comparisons also revealed that differential reliance on conserved molecular pathways modulates species-specific brain development. It has been shown that preferential utilization of the Robo or Dll1 pathways distinguishes the mode of neurogenesis in mammals versus in reptiles and birds (Cárdenas et al., 2018). In particular, in human brain organoids, genetic manipulations that modulate the strength of Robo and Dll1 signaling were sufficient to change the output of neuronal differentiation (Cárdenas et al., 2018). Another conserved signaling cascade that shows species-specific utilization is the mTOR pathway. Transcriptomic analysis of brain organoids derived from multiple primate species revealed a preferential reliance on this pathway in human, compared with chimpanzee and macaque (Pollen et al., 2019). This is consistent with the finding that activation of the mTOR signaling pathway via genetic ablation of *PTEN* resulted in pronounced expansion and folding in human brain organoids (Y. Li et al., 2017). In contrast, *Pten* deletion in mouse PSCs led to a modest increase in brain organoid size and failed to induce structural folding (Y. Li et al., 2017). Together, these studies exemplify the possibilities of using PSCs and brain organoids to investigate the conserved and nonconserved principles of brain development across multiple species. While much remains unknown about the mechanisms that drove the expansion and complexification of the human brain, the availability of these technological implements opens up exciting opportunity for exploration.

Experimental considerations for using brain organoids

Because of the many advantages brain organoids provide, it could be beneficial to incorporate them into study designs in pursuit of a pertinent biological question about neural development, disease, and evolution. However, it is important to note that the current brain organoid technology has yet to succeed in recapitulating many key aspects of human fetal brain. These limitations include incomplete cellular composition and anatomy, lack of synaptic and circuit maturation, as well as high levels of variability. These caveats are to be carefully weighed to determine whether brain organoids are suitable and advantageous in addressing a particular biological question.

The initial considerations of implementing a brain organoid experiment are common for all PSC-based models. While the ability to derive iPSCs from any individual creates an opportunity to examine the underlying biology across diverse human genetic backgrounds, it inevitably generates hurdles in establishing causal correlations between genetic variants (genotypes) and experimental readouts (phenotypes). For many of these instances, inclusion of isogenic controls is a natural solution to exclude variations caused by genetic background differences. Gene editing tools, such as TALEN and CRISPR/Cas9, have made it possible to create isogenic pairs of hPSCs that differ exclusively at the desired genetic locus. Inclusion of more than one parental hPSC line can be helpful in validating genetically penetrant findings. For other studies where a genetically diverse cohort is a necessary starting point, such as investigating polygenic risks associated with neural developmental and degenerative diseases, a sufficiently powered initial population with appropriate clinical stratification should be assembled. From these steps onward, the decision of whether and how to incorporate experiments using brain organoids should be made cognizant of their limitations and guided by the underlying biological question.

Generating the right cell types and structures

Not unlike their 2D counterparts, 3D brain organoids can be generated to assume different regional, temporal, and lineage identity. These methods use developmental cues and patterning factors that guide the differentiation of hPSCs and their primitive neuroepithelial progenies. A critical first evaluation is needed to identify the appropriate organoid derivation method for the biological question at hand. The least restrictive differentiation method relies on the neuralization propensity of hPSCs and generates organoids bearing broad cerebral cortical identity (Lancaster et al., 2013). Addition of morphogens and growth factors generates brain organoids bearing the specific identity of cortex, hippocampus, thalamus, ventral forebrain, midbrain, and hindbrain (Kadoshima et al., 2013; Muguruma et al., 2015; Paşca et al., 2015; Sakaguchi et al., 2015; Jo et al., 2016; Qian et al., 2016).

During *in vivo* development, morphogens and growth factors often exist in limited amounts and in gradients that instruct different signaling responses and contribute to the spatial patterning of the brain. A recent study by Cederquist et al. (2019) generated organoids with inherent positional identity in response to a gradient of sonic hedgehog. This strategy is broadly applicable to model the formation of *in vivo*-like topography in brain organoids and explore long-range signal propagation. As mentioned above, fusion of multiple region-specific brain organoids has been used successfully to study the directional migration of inhibitory neurons (Bagley et al., 2017; Birey et al., 2017; Xiang et al., 2017) and to model reciprocal projections between cortical and thalamic organoids (Xiang et al., 2019).

The choice of differentiation method also depends on the cell types of interest. Studies that require access to early neural precursors can rely on the ability of brain organoids to form the equivalent of early germinal zones encompassing the diverse neural stem and progenitor cells that exist *in vivo*. However, cell types that emerge later during fetal brain development, such as mature neurons whose identities are coupled with their physical positions and functional connections, may not be sufficiently specified within the brain organoids due to the absence of this prerequisite information. Gene expression and electrophysiological studies showed that cell types within the developing brain organoids most closely resemble first and second trimester human fetal brain (Paşca et al., 2015; Qian et al., 2016; Watanabe et al., 2017). The limitation in achieving cellular maturity levels equivalent of postnatal and adult brain is not limited to neurons. Other resident cell types, such as astrocytes and oligodendrocytes, can be generated in brain organoids (Paşca et al., 2015; Madhavan et al., 2018; Sloan et al., 2018; Marton et al., 2019). Their maturation is also protracted and may be difficult to accelerate without a cost to neuronal differentiation using existing methods developed based on 2D directed differentiation (Yang et al., 2013; Tcw et al., 2017; Lager et al., 2018; X. Li et al., 2018; Zheng et al., 2018). We also note that anatomical features, such as the development of a consistent axis, an expanded cortical plate, and its complexification into the stereotypic 6-layer cortex structure, are currently poorly recapitulated in brain organoids. These limitations are not unique to brain organoids, and are instead common to PSC-based modeling efforts where adult cell types and structures are desirable. It remains to be seen whether approaches that have been effective in promoting accelerated maturation of distinct cell types in 2D cultures can be adapted to brain organoids (Zhang et al., 2013; Qi et al., 2017; Yang et al., 2017; Tchieu et al., 2019). Last, a sometimes-overlooked consideration in the choice of organoid derivation method is the desired size and complexity of the 3D structure. Whereas large and complex self-patterned cerebral organoids can be more effective in modeling developmental and disease processes that affect neural tissue expansion and organization, can support the study of long-range signal transduction in 3D space, and permit the investigation of regional interactions, they tend to be more heterogeneous and variable (Lancaster et al., 2013; Velasco et al., 2019). This may limit their use in phenotypic discovery and high throughput assays. Protocols have been developed to generate less variable neural structures with reproducible size, cellular composition (Velasco et al., 2019; Yoon et al., 2019), and even structural symmetry (Zhu et al., 2013; Knight et al., 2018). While some of these approaches may not permit the development of complex features, they can be preferred where variability is less tolerated.

Incorporating the missing cellular and structural components

While the majority of the cells in the human brain come from the neuroectoderm lineage, an important minority population derives from the mesoderm. This includes the vascular endothelial cells and resident microglia. Directed differentiation methods used to convert hPSCs into the neural lineage do not generate these cell types. The endothelial cells and microglia migrate into the early developing brain and codevelop with the resident neural cells. Dysfunction in these cell types is involved in a diverse array of neurological conditions, including Alzheimer's disease, Parkinson's disease, autism, schizophrenia, and cerebrovascular diseases, such as cerebral cavernous malformations (Fischer et al., 2013; Li and Barres, 2018). To model these complex disorders,

strategies to incorporate these missing cell types into 3D brain organoids are required.

The efforts to generate vascularized brain organoids can benefit from the recent development of hPSC-derived blood vessel organoids (Wimmer et al., 2019), potentially allowing codevelopment or fusion of organoids with brain and vasculature identities. While current iterations of blood-vessel organoids are not yet capable of supporting circulation outside a xenograft host, future engineering approaches may facilitate this process *in vitro* via active mechanical perfusion.

Brain organoids are also devoid of microglia, the resident immune cells of the CNS. We (Muffat et al., 2016) and others (Abud et al., 2017; Douvaras et al., 2017; Haenseler et al., 2017; Pandya et al., 2017; Takata et al., 2017; Brownjohn et al., 2018) have recently shown that hPSCs can be differentiated into primitive yolk-sac macrophages, which can then be induced to mature into microglia-like cells sharing many characteristics of developing human microglia. These cells can be cocultured with isogenic neuronal and astrocytic cells in 2D. However, their identity, fine-tuned by the neuroectodermal environment in which they reside, is remarkably labile: *ex vivo* microglia lose their morphology, functional characteristics, and steady-state expression profile. From an experimental standpoint, it is therefore highly beneficial to provide them with neural tissue to reside in, by reaggregating them with isogenic neurons and glia to assemble an organoid with defined cellular components, or letting them invade preformed 3D brain organoids (Muffat et al., 2016, 2018). Their microglial identity on the transcriptomic level is enhanced under these coculture conditions, and they can display physiological characteristics of *in vivo* microglia, including highly branched ramifications, surveying behavior, and responses to cellular damage and infectious agents (Muffat et al., 2016, 2018). It is reasonable to posit that the presence of microglia will in return affect the fate and function of neuroectodermal cells that form the bulk of the organoid. It is worth noting that rare homozygous *CSF1R* mutations in humans lead to loss of microglia and severe developmental malformations, such as callosal agenesis (Guo et al., 2019; Oosterhof et al., 2019). Furthermore, recent findings showed that IL1A, C1Q, and TNF- α secreted by microglia can control the cytotoxicity of astrocytes toward neurons and oligodendrocytes (Liddelow et al., 2017).

Recent studies comparing the transcriptomic profiles of human fetal brain tissues and brain organoids revealed many similarities but also distinct differences (Camp et al., 2015; Qian et al., 2016; Amiri et al., 2018; Pollen et al., 2019; Velasco et al., 2019). The absence of the aforementioned cellular and structural components figures prominently in these differences. Lack of cell types that originate from non-neuroectoderm lineage (e.g., microglia, endothelial cells), elevated metabolic stress due to nutrient and oxygen deprivation, and delayed maturation are among the most noted limitations of the current iterations of brain organoids. These findings highlight the value of continuous research on primary human cells and tissues, to understand human neurodevelopment, and to guide the implementation of more physiological organoid systems.

Navigating the variability of the assays

Brain organoids can be analyzed in a variety of phenotypic assays. They are conducive to canonical *in vitro* approaches that may be difficult *in vivo*, such as live imaging over long developmental time courses. They also have tissue-like density and anatomical features that benefit from handling techniques intended for tis-

sues. For example, histology methods for structural analysis, organotypic slice culture for imaging and electrophysiology, and intraventricular injection of DNA for electroporation have often been used on brain organoids.

A major limitation of many current brain organoid systems is their variability, reflected in the differences in size, shape, cellular composition, and cytoarchitecture among samples generated in the same batch (Kelava and Lancaster, 2016; Heide et al., 2018; Pollen et al., 2019). Approaches that improve the homogeneity of the starting hPSCs or narrow their differentiation trajectory to a defined regional identity can reduce such variability (Velasco et al., 2019; Yoon et al., 2019). The use of Matrigel as an embedding material and media supplement may provide a supportive extracellular matrix and a concentrated source of nutrients. However, this creates batch-to-batch variability due to the undefined nature of Matrigel. In addition, the step of embedding an embryoid body into a droplet of Matrigel leads to variability and introduces organoid-to-organoid differences. Efforts to identify the mechanical properties and molecular components of Matrigel that contribute to organoid growth could help to create more defined culture protocols. A better understanding of the parameters leading to formation of ventricular structures in organoids may illuminate the role of these cerebrospinal fluid compartments during brain development while allowing generation of more reproducibly polarized features. Methods that generate smaller neural structures (Knight et al., 2018) also enjoy higher reproducibility, albeit at the cost of having fewer structural features.

Overall it may seem that the current approach of relying on the self-organizing and self-patterning properties of stem cells necessitates the tolerance of some stochasticity in the system. However, it should be remembered that *in vivo* development, from a single cell to an entire organism, is remarkably reproducible. It is foreseeable that further knowledge in human development, gained through studying hPSC-based models, will guide rational design of better differentiation and engineering strategies to recapitulate more *in vivo*-like features, thus driving iterative advances.

Navigating the scales and throughput

Brain organoid systems provide an opportunity for systematic assessment of cell types and anatomical structures that are otherwise difficult to access during fetal development. Whereas successful high-throughput chemical and genetic screens using hPSCs-derived 2D neural cultures have recently been reported (Xu et al., 2016; Zhou et al., 2017; Y. Li et al., 2019), implementing high throughput assays in organoids may encounter added hurdles. Achieving consistency and reproducibility among individual organoids will be of paramount importance for scaled up productions. Developing assays that can reliably and sensitively detect phenotypic alterations in free-floating 3D structures is another key challenge. We envision that effective strategies may emerge from new engineering solutions as well as approaches adapted from chemical and genetic screens in model organisms, such as *Caenorhabditis elegans*, zebrafish, and planarian.

In conclusion, organoid systems leverage the amazing self-organizing properties of stem cells to re-create complex tissue and organ development in a dish. In the span of just a few years, this emerging tool has already advanced our knowledge of human development, disease, and evolution. As for every nascent technology, the organoid system will continue to evolve. This requires the users of the technology to be cognizant of current limitations and agile in implementing adaptations. We are confident that the combination of genome engineering, uses of gradients to guide differentiation into polarized neural tissues, modular assembly

of regional elements, and inclusion of relevant neural and non-neural cell types will lead to the establishment of improved brain organoid systems.

Response from Dual Perspective Companion Author Kristen J. Brennand

We wholeheartedly agree with Tian et al. that human-induced pluripotent stem cells (hiPSCs) have revolutionized studies of human development and disease. Whether conducted in 2D or 3D models, the integration of hiPSC-based models and CRISPR engineering makes possible the generation of patient-derived brain cells with which to probe the mechanisms underlying brain disease. Overall, hiPSCs are remarkable tools that enable us to move toward precision medicine; however, whether working in 2D or 3D contexts, researchers must carefully design experiments in light of the known heterogeneity between individuals and the variability within and between replicates. The advantage of 2D models is reduced experimental variability and timelines, whereby rapidly generated and standardized cultures are highly amenable to high-throughput assays. Conversely, 3D organoids permit prolonged maturation and increased cellular complexity, promising to improve the physiological relevance of hiPSC-based models. These are complementary rather than competing approaches that together represent exciting new methods to generate, manipulate, and conduct drug screens on otherwise inaccessible cell types for the study of human neurodegenerative and psychiatric disorders.

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