The Emergence of Stem Cell-Based Brain Organoids: Trends and Challenges

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Recent developments in 3D cultures exploiting the self-organization ability of pluripotent stem cells have enabled the generation of powerful in vitro systems termed brain organoids. These 3D tissues recapitulate many aspects of human brain development and disorders occurring in vivo. When combined with improved differentiation methods, these in vitro systems allow the generation of more complex “assembloids,” which are able to reveal cell diversities, microcircuits, and cell–cell interactions within their 3D organization. Here, the ways in which human brain organoids have contributed to demystifying the complexities of brain development and modeling of developmental disorders is reviewed and discussed. Furthermore, challenging questions that are yet to be addressed by emerging brain organoid research are discussed.

1. Introduction

For centuries, developmental neurobiologists were using a variety of deconstructive approaches in vitro or ex vivo to understand the complexity behind the human brain development.1,2 Recent technological advancements in 3D cell culture methods have immensely helped developmental neurobiologists, and it is becoming evident that the field is greatly benefited by the use of 3D human brain organoids. These new classes of in vitro human brain tissues serve as alternatives to existing animal models that fail short in modeling the pathophysiology of human brain developmental disorders. In particular, studying the human brain has been challenging because of the complexity of the processes that regulate its development. Also, applying knowledge from other model organisms to understand the human brain is not always informative because of remarkable differences in its development compared to that of other mammalian species. One of the differences that appear to be unique to the human brain is an increased neuronal density in combination with an expanded cortical region.

Fully recapitulating the inherent complexities of the human brain in vitro is currently scientific fiction. However, this challenge has motivated researchers to develop in vitro culturing technologies that generate functional and physiologically relevant 3D human brain organoids from pluripotent stem cells as an important first step.3 While brain organoids have demonstrated their ability to recapitulate neurogenesis, migration, positioning and layering of cortices,3,5,6,12,13 they still fail to meet certain critical aspects of in vivo, especially when 3D brain organoids are used as models of brain development. For example, organoids have limited growth potential due to inadequate oxygen and nutrient supply, leading to necrosis at the inner core of the organoid. This is due to the fact that organoids lack circulation and that nutrient and oxygen supply is solely based on the diffusion process. Another shortcoming of brain organoids is randomness in brain regions, lack of anterior–posterior, dorso–ventral, and medio–lateral organization of organoid tissues, and poor reproducibility. Despite all of these inherent limitations, 3D human brain mimetics have already offered unprecedented opportunities to understand some of the mechanisms of human brain development and pave light on the pathomechanisms of neurodevelopmental and neuropsychiatric disorders.3,6 In this review, I attempt to highlight the key discoveries that paved foundations to study human brain development in vitro. Another important goal of this review is to provide the current trends in brain organoid research and highlight unanswered questions that can still be addressed with the current state-of-the-art organoid technologies.

2. Self-Organization in Developmental Biology: From Ancient Methods to Current Technologies of 3D Brain Organoid Cultures

One of the fundamental driving processes in the formation of 3D tissues is the self-organization ability of pluripotent cells that are capable of undergoing self-renewal as well as differentiation in response to genetic, chemical, and mechanical stimuli. The phenomenon “self-organization” was experimentally tested as...
early as the mid-20th century. Nevertheless, whether “self-organization” or “induction effects” is the key determinant of developmental cues in tissue morphogenesis has been an intense discussion among developmental biologists. In fact, in 1952, Moscona and colleagues conducted series of experiments and tested the ability of disassociated chick embryonic cells organized into tissue-like aggregates. In retrospect, these seminal reaggregation experiments were the first to reveal that isolated cells have the ability to undergo self-organization to form neural rosettes-like structures, even before that term was used. Weiss and Taylor then studied the ability of disassociated cells from kidney, liver, and skin to organize into 3D aggregates similar to their original tissue counterparts. These elegant experiments have clearly emphasized the phenomenon of “self-organization” as one of the most important aspects in organism development.

The literature in this area may be scant, but, as far as one can survey it, these early experiments largely focused on organ resynthesis that often required a living host organism into which isolated cells were implanted. In other words, per se no pure in vitro experiment was performed in that era to demonstrate the generation of 3D tissues or brain-like tissue starting from homogeneous mass of cells. This could have been due to the limitations of acquiring a sufficient mass of pluripotent cells or the lack of an experimentally tractable 3D culturing system that allowed the spontaneous differentiation of self-renewing stem cells into neurons.

Technological advances in stem cell biology during the late 2000s have satisfactorily unlocked, allowing researchers to generate neural cell lineages from pluripotent stem cells. There are two milestones in this aspect of modern in vitro developmental neurobiology. First is the astonishing discovery by Zhang et al. demonstrating the generation of neural rosettes from embryonic stem cells (ESCs). As can be seen below, I will emphasize that neural rosettes are one of the most important intermediates in the process of generating brain organoids. Second is the discovery of defined reprogramming factors by Takahashi and Yamanaka, who successfully induced pluripotency in a variety of somatic cells.

The technology of induced pluripotent stem cells (iPSCs) has been a paradigm shift as it offered generation of patient-specific stem cells and allowed modeling pathomechanisms in disease-relevant tissue or cell types. Thus, with the advent of pluripotent technologies, many elegant works have reliably reproduced the generation of neural rosettes from embryoid bodies (EBs).

The development of the mammalian nervous system begins with a neural tube consisting of a single layer of epithelial cells enclosing a lumen that then expands to the ventricular zone (VZ) from which neurogenesis begins. Here the progenitor cells are placed along the apicoaxial axis. A similar organization is seen in neural rosettes generated in vitro, which exhibit a strikingly similar architecture to that of the neural tube in which progenitor cells are apicobasally placed such that a lumen-like structure is generated that is similar to VZ in its apical side.

As these methods have mainly used serum-free culture media, they have reinforced the idea of “self-organization” in which pluripotent cells are capable of generating complex neuronal tissues similar to forebrain-derived cortical neuronal networks of its in vivo counterparts without any external cues. Several key discoveries have come from the Sasai laboratory. They essentially differentiated neural rosettes from EBs in a serum-free media and established the so-called “serum-free culture of embryoid bodies” (SFEBs). Importantly, instead of adding serum, Watanabe and colleagues considered specific growth factors to induce the differentiation of forebrain neural progenitors. SFEBq (the next version of their innovation) allowed the generation of more organized and highly polarized neural rosettes with a larger surface area. These developments suggested that the combination of “self-organization” and “induction effect” could lead to more complex neural tissues. Possibly, pluripotent cells first self-organize to establish a kind of primitive structure that serves as a precursor. Upon adding specific factors, the precursor structures then undergo a directed differentiation instructed by the growth factors via an “induction effect.” In parallel, a number of laboratories have advanced the neuronal differentiation conditions to generate mature human neurons in 2D cultures. As an example, the Studer laboratory has developed robust protocols in differentiating midbrain dopamine neurons, which could be maintained over several months. Importantly, these dopamine neurons could functionally be integrated into parkinsonian monkeys. Likewise, their earlier works have defined the methods to differentiate neural crest cells with eventual engraftment into living hosts and evaluated their survival, migration, and differentiation. Taken together, major advances were already pioneered with 2D cultures that have dramatically improved our knowledge on neural differentiation. While none of these experiments employed culturing of neural tissues entirely in 3D as floating objects, they defined differentiation conditions, which lay foundations for the recent 3D cultures.

3. The Emergence of Stem Cell-Based 3D Brain Organoids

To be precise, a paradigm shift occurred only in the last decade by the pioneering efforts by the Sasai and Vaccarino laboratories, which cultured neural cells from 2D to semi-3D (in which neural tissues were plated first and then moved to suspension cultures) or fully 3D suspension cultures. They generated self-organized structures from human pluripotent cells recapitulating several aspects of telencephalic development. These 3D structures took 50–100 days to exhibit tissue-like morphologies, which the authors named “neural epithelial tissues.” These neural tissues harbored polarized radial glia, intermediate progenitors, and layer-specific cortical neurons similar to the cytoarchitecture of their in vivo tissues. It was then Lancaster et al. who made a quantum leap in optimizing 3D cultures by utilizing Matrigel to embed neural tissues and growing them in spinning bioreactors that caused an enhanced diffusion of nutrients to embedded tissues. This was a key discovery that led to the production of “mini brains,” structures that she called “cerebral organoids.” These cerebral organoids grew up to ≈4 mm in diameter within 60 days. These floating objects stunningly recapitulated several aspects of developing human brain. Importantly, Lancaster’s method is the combination of serum-free EB culturing without any “induction signals.” Due to the absence of any inductive signals, the generated cerebral organoids not only contained primitive cortical plate but several regions similar to primitive forebrain, hindbrain, and midbrain. Furthermore, specific cell types in these organoids were spatially restricted such that
Apical progenitors were aligned to form a lumen that is similar to VZ of mammalian brain and cortical neurons were positioned basally to the VZ, forming a cortical plate.

Further efforts by Gabriel and colleagues\[4,5,36\] have fine-tuned the culturing method to generate organoids within 23 days, which were named “brain organoids.” By enhancing the homogeneity of organoids, the authors intended to reliably recapitulate the defects observed in brain organoids derived from microcephaly patients. In their protocol, the induction of neural differentiation to form neural epithelia starts directly from iPSCs and not from EBs. The EB formation step was skipped in order to continue neural differentiation in a controlled and directed manner, thereby limiting the spontaneous and undirected formation of other germ cell layers, such as mesoderm and endoderm. The organoid medium in their method used dorsomorphin and SB431542. Dorsomorphin is a small-molecule inhibitor of bone morphogenic protein (BMP) and SB431542 inhibits the transforming growth factor-β (TGF-β)/activin/nodal signaling pathway. Two more additions were made on top of the methods that generate brain organoids, which were essentially to inhibit the SMAD signaling pathways that promote region-rich organoids enriched with diverse kinds of neurons defining dorsal cortex, forebrain, midbrain, and hypothalamus. Generating these organoids again combined “self-organization” (brain organoids from EBs) and “inductive effects” (inhibiting SMAD pathways).\[37,38\] All together, these efforts have shifted our views of using brain organoids as alternative model system to address unanswered questions in developmental biology and modeling certain disorders that cannot be convincingly addressed using conventional animal models (see below). I summarize the historical milestones that led to the generation of brain organoids in Figure 1.

### 4. 3D Brain Organoids to Decode Early Events of Human Brain Development and Developmental Disorders

The recent, favorable technological advances in 3D brain cultures enable developmental biologists to discover the mechanisms of human brain development and to model...
neurodevelopmental disorders. Since human brains differ from those of other animals, organoids could fill the gap in our knowledge between humans and other model organisms. This might lead to the possibility of replacing the use of laboratory animals to study the biology of human brain. The developing brain organoid cultured up to 30 days recapitulating early events of human brain development (early brain organoids or first-generation organoids) is already a suitable system that fulfills this criterion in decoding the pathomechanisms of primary microcephaly.

Primary microcephaly is a neurodevelopmental disorder caused by mutations in centrosomal genes, which affect a patient’s head circumference, making it smaller than normal. This is because the brain does not develop normally, perhaps due to depletion of neural progenitor cells (NPCs). However, this speculation has remained untested, up until now, due to the lack of suitable human models. When this hypothesis was tested in rodents, the observed phenotypes were minimal or not comparable with that of human microcephaly. An example of this is knockout mouse models of Nde1, which causes a mild form of microcephaly. In comparison, human mutations in Nde1 caused severe microcephaly with significantly reduced cortical surface. Patient-derived brain organoids have been used to show that premature differentiation of radial glial cells is one of the mechanisms that cause the depletion of NPCs at the VZ where neurogenesis begins.

Mechanisms behind the depletion of NPCs in developing brain remain unknown. Analyzing the kinetics of radial glial cells’ division planes at the ventricular lumen of developing brain organoids revealed that their division planes are mostly oriented horizontally (in other words, vertically oriented cleavage plane) to the surface of VZ and also exhibited fractions of cells whose division plane is oblique and vertical orientation. The presence of major fractions radial glial cells (RGs) with a horizontally oriented division plane makes the most sense that they have to expand symmetrically to generate a sufficiently large pool of neural stem cells in order to generate a normal sized brain. Intriguingly, a similar distribution dynamic is indeed observed in the human embryonic brain. This observation lends further credence to the idea that human brain organoids are the closest models to the human brain. It is particularly important to emphasize that RG division plane trends in rodent models are less dynamic in terms of the rotation of the division plane. Altogether, these results show that organoid tissues offer a unique opportunity to measure symmetric versus asymmetric division of neural stem cells in its 3D volume—a key aspect that is fundamental to understand early neurogenesis.

It remains unknown if brain organoids derived from microcephaly patients would exhibit an altered orientation of the division plane of the radial glial cells. Indeed, analyzing at least two independent microcephaly organoid models (mutations in CDK5RAP2 and CPAP) showed a majorly disrupted trend of radial glial cells’ division planes with major proportions of cells orienting vertical or oblique to the ventricular surface, highlighting that neural stem cells fail to undergo symmetric expansion.

Compared to nonhuman primates, the human brain differs at various stages of development. For example, it has an increase in neuronal density, more diverse types of progenitors, an extended cortical network, and more cortical folding proportional to its size. For these reasons, human brain development takes a relatively very long time, including various postnatal developmental stages. For example, human cerebral cortex development requires a massive expansion of the progenitor pool at the VZ, which eventually differentiates into glutamatergic neurons and migrates to the primitive cortical plate. These neurons then travel within in the cortex from the lower to the upper layer of pia mater. Similarly, inhibitory GABAergic neurons also migrate into the cerebral cortex. Such cortical circuits are not simple processes of connectivity between different neuronal types but via a precise proportion of neuronal integration coupled with long distance migration from ventral forebrain to dorsal pallium. Defects in interneuron migration contribute to epilepsy and neuropsychiatric disorders. Addressing these complexities is another challenge that requires advanced methods of culturing 3D brains. In a pioneering study, the Pasca laboratory grew organoids for a prolonged period of time resulting in larger aggregates. These were called organoids, which can be thought of as second-generation organoids (Figure 1). These organoids exhibited distinct layers of cortex and resembled the mature postnatal stage of human brains. Further developments on these cultures were made to generate region-specific tissues resembling the ventral and dorsal pallium. When these tissues were fused together, it was possible to observe how interneurons migrate and precisely integrate to form circuits. The Park laboratory elegantly performed similar corticogenesis studies in which they could model the development of medial ganglionic eminence (MGE). Fusing MGE and cortical organoids nicely recapitulated human interneuron migration and integration. Similarly, Quadro et al. cultured organoids for more than nine months to generate organoids exhibiting mature features exhibiting neuronal networks and dendritic spines. Furthermore, their extensive work on sequencing many thousands of cells derived from independent organoids revealed the presence of a wide diversity of cells that are relevant to the mature brain. Thus, these organoids should help addressing questions beyond the early developmental stage at the progenitor level. Collectively, to date, these first-generation (early organoids recapitulating early events of brain development) and second-generation organoids (mature organoids capable of recapitulating later stages of brain development) are the fundamentally important and powerful in vitro systems to understand human brain development, maturation, and disorders.

5. Region-Specific 3D Brain Organoids

Building on the above systems, several types of brain organoids have been generated using inhibitors of specific pathways to induce the formation of region-specific organoids. These region-enriched organoids were instrumental to address specific aspects of human brain development (summarized in Tables 1 and 2). For example, retinal morphogenesis is elegantly described by the extraordinary ability of pluripotent cells to self-organize into complex structures in vitro. Retina morphogenesis a multifaceted process, generating stratified neural epithelia from neuroectoderm. The pioneering work of the Sasai laboratory has uncovered these astonishing properties of pluripotent cells. Although cultivating retinal organoids consumes substantial amount of time...
Table 1. Brain organoids to study in vitro neurobiology and disease modeling of neurodevelopmental disorders.

| Type of organoid       | Aspect of neurobiology studied                                                                 | Disease modeled/potential application                                                                 | Mechanisms revealed                                                                 | References |
|------------------------|------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|------------|
| Cerebral/early brain organoids | - Neural progenitor maintenance  
- Corticogenesis  
- Neuronal migration                                                   | - Genetically caused microcephaly  
- Zika virus mediated microcephaly  
- Neural progenitor depletion and cell death                             | - Premature differentiation of neural progenitors  
- Neural progenitor depletion and cell death                               | [4-6,51] |
| Midbrain organoids     | - Differentiation of dopaminergic neurons and neuromelanin-containing neurons                             | - Potential to model Parkinson’s disease                                                                 | - Not yet used for disease modeling                                                 | [52,53] |
| Hypothalamus organoids | - Differentiation of hypothalamic neurons and POMC-positive peptidergic neurons                        | - Potential to model hormonal and metabolic disorders including Prader-Willi syndrome                  | - Not yet used for disease modeling                                                 | [58] |
| Adenohypophysis organoids | - Generation of Lim3-positive pituitary progenitors                                                  | - Potential to model pituitary dysfunction                                                                 | - Not yet used for disease modeling                                                 | [54] |
| Hippocampus organoids  | - Dorsomedial telencephalon development                                                              | - Potential to model cognitive dysfunctions due to Alzheimer’s disease                                | - Not yet used for disease modeling                                                 | [53] |
| Cerebellum organoids   | - Cerebellum development                                                                             | - Potential to model SCA and Dandy-Walker syndrome                                                    | - Purkinje neurons are vulnerable to triiodothyronine depletion                    | [56,57] |
| Dorsaltelencephalon organoids | - FOXG1-positive forebrain development                                                                | - ASD                                                                                                 | - Imbalance between GABAergic and glutamatergic neuronal fates                    | [14] |
| Forebrain assembloids  | - Interneuron migration and circuit formation                                                         | - Timothy syndrome                                                                                    | - Abnormal saltatory migration                                                      | [3] |
| Photosensitive organoids | - Vast diversity of cells and network activities of neurons                                          | - Potential to model disorders of mature brain                                                         | - Dendritic spines and spontaneously active neuronal networks                     | [11] |
| Retinal organoids      | - Retinal pigment epithelia development                                                              | - Neuroretinal disorders and retinal dystrophy due to ciliary defects                                 | - Impaired ciliogenesis                                                             | [58] |

ASD, autism spectrum disorders; POMC, pro-opiomelanocortin.

up to 150 days, several laboratories have successfully generated optic cups or retinal organoids harboring retinal pigment epithelia and functional photoreceptors.

Midbrain-like organoids, which encapsulate the properties of the substantia nigra, are a useful model for Parkinson’s disease. This particular type of organoid has been cultured with Wnt activators and SMAD inhibitors. This caused an enhanced differentiation to neuroectodermal lineages resulting in structures that resemble the midbrain floor plate. Further treatment with neurotrophic factors generated dopaminergic neurons expressing tyrosine hydroxylase. Likewise, inhibiting TGF-β pathways in developing neuroepithelium generated hippocampal-like organoids. BMP-4 ligand and Wnt agonists were then used to induce dorsomedial lineages differentiated from neuroepithelium, leading to the formation of choroid plexus-like tissues (expressing Lef1, Lix 2, and Pax6-positive cells). These tissues could recapitulate some of the aspects of dorsomedial telencephalon. Thus, further development of the hippocampus-like complex region of human brain could help model cognitive dysfunctions associated with Alzheimer’s disease. To generate organoids that contain regions resembling the hypothalamus, first SMAD, BMP, Nodal, and activin signaling pathway inhibitors were used to induce the formation of structure that resemble the anterior regions of the brain. Hypothalamic lineages were then induced when such structures were treated with Shh, Wnt3a, and purmorphamine. For cerebellar organoids, growth factors such as insulin and FGF2 and a TGF-β receptor blocker were utilized to differentiate pluripotent cells into neuroectodermal lineages with simultaneous inhibition of a mesenchymal cell fate. This was sufficient to induce the expression of midbrain-hindbrain (MHB) markers of BX2 and EN2 followed by an elevated level of Wnt1 and FGF8, which are implicated in the generation of the isthmus organizer at the embryonic MHB. To generate functional Purkinje cells, FGF19 was used to trigger the differentiation of neuroepithelium into structures that are dorsoventrally polarized and resembles the neural tube. Supplemented growth factors such as SDF1 and FGF19 promoted the generation of a cerebellar plate neuroepithelium with a rhombic lip-like structure at one end that has a three-layer cytoarchitecture similar to the cerebellum. Importantly, these regions harbored neuronal cell types positive for calbindin, aldolase, pavalbumin, neurogranin, and gamma-aminobutyric acid (GABA), all of which are markers of mature Purkinje cells.  

Cerebellar organoids are unique in modeling spinocerebellar ataxia (SCA), a hereditary, progressive, and degenerative disorder.
Typical brain organoids develop solely based on presence of cellular diversities, complex interactions, and term culturing of organoids, which eventually revealed the brain development. Indeed, progress has been made in a prolonged period of time. This hampers the studies related to culturing is limitations in keeping the 3D tissues fully alive over early brain, which enables studies related to early neurogenesis.

Organoids vastly majority of fundamentally vital questions in developmental vitro is greatly anticipated. While this seems to be obvious, a ble 1). With this magnitude of progress so far, there is no doubt vitro systems to understand disease mechanisms ranging from more, organoids have proved to be physiologically relevant in into the mechanisms of human brain development. Further-

- Midbrain organoids: Wnt activators and SMAD inhibitors
- Hypothalamus organoids: Inhibitors that blocks TGF-β pathways
- Adenohypophysis organoids: DAPT, SAG, BIO, BMP4, dorsomorphin, Wnt4, Wnt5, FGF8, Nodal, iWP2
- Hippocampus organoids: Wnt inhibitor IWR1e, TGF-β inhibitor SB431542, 10% FBS, GSK3 inhibitor CHIR99021, BMP4
- Cerebellum organoids: SB431542, FGF2, FGF19, SDF1
- Dorsal telencephalon organoids: Noggin, FGF2, rhDKK1, EGF, ascorbic acid, BDNF, GDNF, cAMP
- Forebrain assemboids: Dorosomorphin, SB431542, FGF2, EGF
- Photosensitive organoids: BDNF
- Retinal organoids: IWR1e, Matrigel, 10% FBS, SAG, CHIR99021, retinoic acid
- Hypothalamus organoids: SMAD, BMP, Nodal and activin signaling pathway inhibitors
- Cerebellum plate neuroepithelium: FGF2,4,8, SAG, retinoic acid, BDNF, GDNF, NT3

| Table 2. Summary of signaling molecules used to generate region-specific brain organoids. |
|---------------------------------------------------------------|
| Type of organoid generated       | Cultured with                                      | References |
| Midbrain organoids              | Wnt activators and SMAD inhibitors                 | [32,33]    |
| Hypothalamus organoids          | Inhibitors that blocks TGF-β pathways               | [35]       |
| Adenohypophysis organoids       | DAPT, SAG, BIO, BMP4, dorsomorphin, Wnt4, Wnt5, FGF8, Nodal, iWP2 | [34]       |
| Hippocampus organoids           | Wnt inhibitor IWR1e, TGF-β inhibitor SB431542, 10% FBS, GSK3 inhibitor CHIR99021, BMP4 | [35]       |
| Cerebellum organoids            | SB431542, FGF2, FGF19, SDF1                         | [56,57]    |
| Dorsal telencephalon organoids  | Noggin, FGF2, rhDKK1, EGF, ascorbic acid, BDNF, GDNF, cAMP | [14]       |
| Forebrain assemboids            | Dorosomorphin, SB431542, FGF2, EGF                 | [3]        |
| Photosensitive organoids        | BDNF                                               | [11]       |
| Retinal organoids               | IWR1e, Matrigel, 10% FBS, SAG, CHIR99021, retinoic acid | [38]       |
| Hypothalamus organoids          | SMAD, BMP, Nodal and activin signaling pathway inhibitors | [38]       |
| Cerebellum plate neuroepithelium| FGF2,4,8, SAG, retinoic acid, BDNF, GDNF, NT3      | [70]       |

BDNF, brain-derived neurotrophic factor; BMP, bone morphogenetic protein; cAMP, cyclic adenosine monophosphate; EGF, epidermal growth factor; FBS, fetal bovine serum; FGF, fibroblast growth factor; GDNF, glial cell-derived neurotrophic factor.

6. Expectations from the Current State-of-the-Art Organoids

As a result of extraordinary progress made within the last five years in culturing 3D organoids, we have gained useful insights into the mechanisms of human brain development. Furthermore, organoids have proved to be physiologically relevant in vitro systems to understand disease mechanisms ranging from microcephaly, neuropsychiatric to neurotropic infections (Table 1). With this magnitude of progress so far, there is no doubt that coming years will be fruitful for developmental biologists and the generation of much more advanced brain organoids in vitro is greatly anticipated. While this seems to be obvious, a vast majority of fundamentally vital questions in developmental biology still remain to be addressed (Figure 2).

6.1. Long-Term Culturing of Brain Organoids

Typical brain organoids develop solely based on diffusion processes generating ventricles whose structures mimic the early brain, which enables studies related to early neurogenesis. One of the major technological shortcomings in organoid culturing is limitations in keeping the 3D tissues fully alive over a prolonged period of time. This hampers the studies related to the neuronal maturation process occurring at the later stages of brain development. Indeed, progress has been made in long-term culturing of organoids, which eventually revealed the presence of cellular diversities, complex interactions, and neuronal networks. Nevertheless, deficiency in oxygen and nutrient supply due to the lack of vascularization would strongly perturb cell survival and overall tissue architecture. To test this aspect, Mansour et al. implanted brain organoids into the mouse brain. Via this experiment, the authors demonstrated the compatibility between two different tissues and the ability of the host vascular system to invade into the organoid to provide better nutrition. Importantly, this procedure has resulted in neuronal maturation in organoids. Pham et al. took an alternative approach by coculturing organoid. Surprisingly, this approach has resulted in vascularization within organoids even before implanting them into a host mouse.

While these approaches suggest that vascularization is beneficial for organoid maturation, intracerebral transplantation of brain organoids in mice is a laborious task and requires special expertise. A recent work by the Lancaster laboratory has piloted an approach fully in vitro to improve nutrient and oxygen supply to eventually improve neuronal survival for a period of several months. The authors named this approach as air-liquid interface cerebral organoids (ALICOs) in which they grew organotypic slices of cerebral organoids and showed an in vivo-like axon guidance behavior. Long-term culturing of organoids that could improve neuronal survival is indeed a high priority for a number of reasons. Perhaps, one of the most important reasons is to use organoids for late onset disorders such as neurodegeneration. Modeling late onset diseases such as neurodegeneration is severely hampered with the current status of organoid culturing technologies, which does not essentially generate mature neurons that mimic physiological status of cell types that appear in adult life. The coming years will hopefully advance the technologies so that the general usage of brain organoids for neurodegenerative diseases can be realized.

6.2. Axes Determination, Pattern Formation, and Interorgan Interactions within Brain Organoids

The current state-of-the-art, organoid-culturing techniques are already sufficient to address these pressing questions. One challenge is how to control the axes of pluripotent cells such that the pattern forms in 3D? This appears to be an important problem because developmental biologists think that, unlike a human
embryo, brain organoids are not able to strictly follow self-patterning rules. As a result, brain organoids often lack precise anterior–posterior and dorsal–ventral axes. This is one of the major reasons for heterogeneity among organoids meaning they are unsuitable to study circuits and connectivity with a 3D organization. For example, it is unclear if whole brain organoids establish anterior–posterior and dorsal–ventral axes. Indeed, a recent work by the Studer laboratory demonstrated that when artificially induced brain organoids can generate gradients of signaling molecules to define spatial topography of forebrain.[65] While this approach has strongly suggested that organoids can be instructed to establish in vivo-like topography, whether the self-assembling property of brain organoids can spontaneously establish such patterns and generate structures associated with forebrain region such as optic cups is not explained in depth, but it has been shown that whole brain organoids generated by Lancaster already showed the presence of pigmented eyecup-like structures embedded as part of the organoid. It remains unknown if this eyecup structure is connected to the forebrain-like region expressing FoxG1-positive cells and if this region also harbors retinal progenitors that express Rax1.

During embryogenesis, the retinal anlage develops laterally from the diencephalon, protruding as an optic vesicle. Its distal part then invaginates to assemble into the optic cup. Thus, in theory, brain organoids should be able to generate functional eyecups constituting retinal pigment epithelium and neurosensory layers of the neural retina. Indeed, substantial work has demonstrated the surprising ability of pluripotent cells to form retinal cells and photoreceptors including a retinal pigment epithelium. In the future, we may see progress toward making hybrid organoids constitute bilaterally symmetric eyecups embedded within a forebrain-like region. When this becomes real, one should be able to test interorgan interactions between connectivity between the ganglionic cell layer of the neural retina and the brain (Figure 2).

6.3. Personalized Brain Organoids for Disease Modeling and Disease Correction

Early brain organoids displaying layered VZ with RGs are already excellent in vitro systems to model developmental disorders such as lissencephaly and microcephaly. Both of these disorders are largely associated with impaired migration or premature differentiation of RGs. To date, only a couple of microcephaly brain organoids have been generated from patients carrying mutations in CDK5RAP2
and CPAP. Mutations in at least 20 different centrosomal/ciliary genes are directly linked to microcephaly, whose pathomechanisms remain yet to be addressed. Experiments with patient-specific brain organoids will uncover whether there are distinct or shared mechanisms causing primary microcephaly. Besides microcephaly, there are a number of developmental disorders whose pathomechanisms remain incompletely explored because of the lack of proper human in vitro models. For example, Rett syndrome, fragile X syndrome, and Cockayne syndromes could be modeled using patient-specific brain organoids. These efforts might help identify disease mechanisms and potential therapeutic targets (Figure 2). Eventually, these studies might help identify the roles the mutant genes have in human brain development.

Finally, the recent technological revolution with genome tailoring such as CRISPR–Cas9 has allowed the researchers to manipulate, introduce, or correct disease-relevant mutations in diverse cell types.[66,67] Of note, CRISPR–Cas9-based genome editing has not been sufficiently utilized in brain organoids either to generate or to correct patient-derived mutations that are implicated in developmental disorders. It is plausible that there is a technical challenge in obtaining stably manipulated, pluripotent cells as an initial step to generate brain organoids. Nevertheless, the combination of genome tailoring and organoids will be a powerful tool certainly and will allow us to generate brain organoids to conduct a functional analysis of genes and to deconstruct human brain development entirely in vitro.

6.4. Brain Organoids for Cancer Cell Invasion and Drug Discovery

Besides using brain organoids for disease modeling, perhaps the most useful aspect of 3D brain organoids is to optimize them for semi-high-throughput drug discovery experiments. One urgent question is to understand the invasive behaviors of patient-derived glioblastoma multiforme (GBM) cells. Glioblastoma is the most frequent malignant primary brain tumor. Despite surgical resection, its rapid growth, resistance to chemotherapy, and high invasiveness mean that GBM patients succumb to the disease with a median survival time of 15 months. Studying its invasive behavior in vitro could help disease prognosis and make a therapeutic decision in a timely manner. The current state-of-the-art is to inject patient-derived GBM cells into the mouse brain and study the outcome. While this approach has provided useful insights, it is time consuming and may not sufficiently recapitulate the actual cellular pathologies occurring in the human brain. Thus, the restricted availability of a suitable in vitro human model that can reliably recapitulate complex human brain development is a significant bottleneck that limits the translation of basic GBM research to a clinical application. This gap can potentially be bridged by the use of 3D brain organoids because it can provide a near-native substrate to study the invasive behavior of GBM cells. Indeed, brain organoids have been already utilized to study the brain tumorigenesis by overexpressing oncogenic mutations.[68] While this study already proves the potential of brain organoids to help understand tumorigenesis, variations in expression levels of oncogenes from organoid to organoid requires further refinement of the methodology. Ogawa et al.[69] devised an elegant method to study the invasion behavior of patient-derived glioblastoma cell lines in brain organoids and identified that GBM cells have the ability to invade within the 3D neuroepithelium. This method basically establishes human brain organoids as a platform to further characterize the invasive behavior of patient-derived GBM cells. Further refinement of this approach possibly converging tissue-clearing and whole mount imaging will certainly be an important tool to categorize invasive behavior of GBM cells depending on their subtypes and genetic backgrounds.

Another challenging and important task is to establish an autologous GBM invasion assay in which the invasive behavior of patient-derived GBM cells within brain organoids derived from the very same patient can be assayed. Accomplishing this task requires establishing robust reprogramming methods combined with thoroughly scaled-up procedures of organoid generation in a reasonable period of time. Hopefully, future years will see these experiments occurring in real. Successfully establishing the GBM invasion assay will help assessing the invasive behavior and neurotoxicity of an individualized GBM and lay foundations for a precision medicine model for testing drugs against tumor invasion (Figure 2).

7. Conclusions and Outlook

This is an exciting era for in vitro developmental neurobiology, especially since many technical advances have emerged within the last decade ranging from culturing brain organoids in 3D, ex vivo implantations to genomic tailoring. While combinations of these methods provide auspicious moments for neurobiologists, one must be selective about questions and choose the right combination of methods. As mentioned before, we have several unaddressed intriguing questions which do not necessarily wait for more technological developments to come in 3D cultures. This is particularly true, considering the questions related to progenitor biology, neurodevelopmental disorders, and neuropsychiatric disorders due to defects in interneuron migration. In other words, we are still at the tip of the iceberg and lag phase of the growth curve. The coming years will place us at the exponential phase of the curve when the power of 3D brain organoids is fully exploited to address the right questions in solving the mysteries behind human brain development and its disorders.

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Conflict of Interest

The author declares no conflict of interest.

Keywords

3D cultures, assembloids, brain organoids, induced pluripotent stem cells (iPSCs), microcephaly, self-assembly
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