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Brain organoids as a model system for human neurodevelopment and disease

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Abstract

The ability to reproduce early stages of human neurodevelopment in the laboratory is one of the most exciting fields in modern neuroscience. The inaccessibility of the healthy human brain developing in utero has delayed our understanding of the initial steps in the formation of one of the most complex tissues in the body. Animal models, postmortem human tissues and cellular systems have been instrumental in contributing to our understanding of the human brain. However, all model systems have intrinsic limitations. The emerging field of brain organoids, which are three-dimensional self-assembled multicellular structures derived from human pluripotent stem cells, offers a promising complementary cellular model for the study of the human brain. Here, we will discuss the initial experiments that were the foundation for this emerging field, highlight recent uses of the technology and offer our perspective on future directions that might guide further exploratory experimentation to improve the human brain organoid model system.

Keywords

Brain organoids; human neurodevelopment; Zika virus; neurotrophic viruses; neurological genetic disorders; autism; brain tumor

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CONFLICT OF INTERESTS

Dr. Muotri is a co-founder and has an equity interest in TISMOO, a company dedicated to genetic analysis focusing on therapeutic applications customized for autism spectrum disorder and other neurological disorders with genetic origins. The terms of this arrangement have been reviewed and approved by the University of California San Diego following its conflict of interest policies.

Introduction

Almost every living organism in the animal kingdom is controlled by the most complex and least understood organ: the brain. Thus, the ability to reproduce early stages of brain development in a dish in the laboratory is one of the most exciting fields in modern neuroscience. Human brain organoid development is an emerging field and has beomce a promising *in vitro* technique; the scientific community has been able to make advances in three-dimensional (3D) modeling using pluripotent stem cells that further our knowledge of many neurological diseases[1]. The basis of our current knowledge about the early stages of human brain development has mostly come from animal models, such as rats and cells grown in a petri dish, or two-dimensional (2D) models. The *in vitro* models are a limited but represent existing alternatives that can partially compensate the lack of access to tissues during early developmental phases in the human uterus. Of course, several studies have been conducted on the human brain, primarily on postmortem tissue[2]. Through these studies, we have learned much about the neurodevelopmental anatomy of the human brain, but the electrophysiological aspects remain unexplored. Animal models have also shed light on many mysteries of the brain, even though they cannot recapitulate the intricacies of the human brain due to distinct evolutionary trajectories[3].

Scientists can now manipulate stem cellsto induce maturation into several specialized cell types using a specific combination of factors that influence cell fate determination. Embryonic Stem Cells (ESCs) have uncharted potential in regard to development. ESC's can be grown in culture dishes for months and maintain the ability to form cells ranging from muscle to nerve to blood cells. This versatility allows derived cells to be used in many scientific studies. Human-induced Pluripotent Stem Cells (iPSCs) are derived from somatic tissue cell types, such as fibroblasts in skin or blood cells, that have been reprogrammed back to an embryonic-like pluripotent state[4]. These iPSCs can be potentially differentiated into any cell type in the human body, enabling the development of specialized human cells in large quantities for therapeutic or research purposes[5].

An organoid is a 3D structure derived from pluripotent stem cells, which are adult stem/progenitor cells that spontaneously self-organize into adequately differentiated functional cell types, and can recapitulate at least some function of the target organ[6]. A human cerebral organoid is described as a group of cells that dynamically self-organize into structures containing different cell types that resemble some aspects of the fetal brain [7,8]. Human brain organoids can be used to study early stages of neural development. Neurons in brain organoids can connect and make simplified, organized neural networks, eventually leading to the developmental steps that all human brains take[9]. By observing the neural development *in vitro*, normally or in a disease context, scientists may be able to uncover the molecular and cellular mechanisms that are necessary for proper development of the human brain[10].

Organoid humble beginnings

Scientists have been on a quest to imitate organogenesis outside the body for more than a hundred years. The practice of explanting pieces of an organism and culturing these grafts to observe the behavior of the cells first occorred in 1906 when Ross Harrison developed the

hanging drop tissue culture technique[11]. Harrison was experimenting with a fragment of an embryo nerve cord that was placed on a drop of lymph and then inverted and sealed. This experiment was the beginning of a study to learn about the origin of the nerve fibers [12]. Placement of the fragment of the embryo nerve cord in the lymph provided an adequate environment for growth and development of the nerve fiber. The conditions set forth by Harrison were not perfect, and thus, other researchers have further adapted this system to culture tissues of diverse origins for prolonged periods [11,13,14]. The adjustments made by the scientific community allowed the development of stable protocols to preserve tissue grafts for an extended period. These protocols led to advancements in studies concerning various physiological pathways that occur within these organs. Eventually, a growth medium was developed that removed the obstacle of repeated tissue acquisition [15,16]. These protocols would later become the first stepping stone into a period when tissue cultures would be popularized in the scientific field.

The *in vitro* culture method has since been used to grow many different types of organoids, but significant barriers would still need to be overcome in the field of neuroscience field. Even though adult and embryonic neuronal cultures were problematic due to the laboriousness of maintenance of the cells [17], some neural processes, such as cell migration [18] and identification of axon cues [19] were possible to stimulate with the isolation of target tissues. A clonal neuronal cell line was eventually derived from mouse neuroblastoma that allowed for more consistent production of neurons, removing the need for constant tissue explants [20,21].

Nevertheless, another bottleneck existed with the *in vitro* culture research model: the inability to accurately model events such as timed neurogenesis. This problem was solved with the use of embryonic stem cell lines that could be directed into producing *in vitro* cultures that better-modeled cell fate conversions in a dish [22,23,24]. Scientists found that the use of specific signaling molecules added ectopically to the medium could produce specific cell types *in vitro*, including neurons found in the central and peripheral nervous system[25,26]. For example, by treating human neural progenitor cells with dual inhibition of transforming growth factor β (TGFβ)/bone morphogenetic protein (BMP) pathways and glycogen synthase kinase 3 (GSK-3), it is possible to enrich the cellular population for cortical neurons. Using protocols with patterning factors led to the development of a more reliable and efficient differentiation protocol for cell specificity. Even with these culture breakthroughs, the complexity of the human brain makes modeling the organ in the laboratory one of the biggest challenges for stem cell scientists.

2D and 3D cellular models

Cell culture is the process by which cells are grown in conditions outside of their regular environment. This technique is used in a wide variety of laboratories, such as in vaccine production, cancer research, and protein therapeutics[27]. There are typically two types of cells used in laboratories: primary cells and established cell lines.

Primary cells are cells that are isolated directly from an organism’s tissue; if isolated and cultivated correctly, primary cells are still able to grow and proliferate with limited

potential[28]. Primary cells are only able to replicate a finite number of times because to the telomeres in DNA are reduced each time the cell replicates[29]. An established cell line is a permanently established cell culture that will proliferate indefinitely given the appropriate conditions. Established cell lines can be derived from clinical biopsies or created by transforming primary cells, usually with viral oncogenes or chemical reagents[30,31]. Both of these cells types can typically be cultured under two main growth conditions: either in adherent or suspension cultures. The medium in which cells are cultured is a standard mix of amino acids, inorganic salts, and vitamins, sometimes with the inclusion of undefined Fetal Bovine Serum (FBS) [32]. FBS typically includes lipids, growth factors, and macromolecules.

In 2D cell culture, monolayer of cells is typically formed as described earlier, while a 3D culture allows the cells to organize themselves into more complex structures that might resemble the tissue of origin. Typically, 2D cell culture is carried out within a petri dish in which the flat surface provides mechanical support for cell adherence. This support allows the cells to form 2D monolayers [33]. The uniformity of a monolayer ensures equal distribution of nutrients and growth factors present in the medium, which encourages homogenous growth [34]. Although uncommon, it is possible to somewhat control the structure of 2D cell cultures through the use of adhesive cell islands, micropillars, and customized microwells[35]. However, this practice is not commonly used because it might affect the apical-basal polarity of the cells, resulting in an alteration of the function of the native cells. For example, a lack of reproducibility in some 2D cultures increases the cost and failure rate of new drug discovery and clinical trials, for example. The cell growth medium can also cause problems. A standard 2D culture consumes medium and exudes waste, which can result in toxic molecules that can ultimately affect the culture environment[32]. Despite these disadvantages, 2D cell cultures are still widely used due to their low cost and straightforward readout analysis methods, such as cell morphology or migration pattern. A variety of cellular phenotypes can be analyzed *in vitro*, and all are heavily influenced by the biochemical and biomechanical environment [36]. Increasing evidence shows that the 2D system *in vitro* response differs from the *in vivo* response to the same stimulation and 3D models could better allow scientists to recreate the natural environment in which these cellular behaviors occur [36].

A typical example is the specific characteristics of cancer cells, which cannot be appropriately modeled in 2D cultures [37]. To overcome the 2D culture limitations, novel 3D cell culture platforms are being created and standardized in order to better replicate *in vivo* conditions [38,39,40]. Differences in 2D and 3D culture system have also been observed for neurodevelopmental disorders. A layered 3D system was used to assay cell migration and maturation of neural progenitor cells derived from Rett syndrome (RTT) iPSCs[41]. The 3D platform was able to reveal subtle but significant dysfunction in neuronal migration and maturation that was not observed in previous traditional 2D monolayer culture experiments[42].

For 3D cell cultures, the makeup and distribution of the cell-extracellular matrix (ECM) and cell-cell interactions might influence the cellular functions. In contrst with monolayers, aggregation of cells in 3D structures creates a gradient of nutrient intake [32]. The 3D models can be more complicated due to the interaction that occurs between the different cells of the

target organ [35]. For example, our organs have a protective barrier composed of epithelial cells to maintain a constant environment that is difficult to recapitulate *in vitro*. The representation of the barrier-like tissue might be more relevant in 3D cultures. Several experimental platforms are being used to reveal the increasing complexity of the ECM around cells, which can have a considerable influence on cell proliferation, differentiation, and longevity of the cells [43,44,45]. Finally, the 3D environment allows cells within brain organoids to grow and expand in multiple directions, forming specific niches that favor fate specification. In fact, single-cell sequencing has revealed the transcriptional identity of distinct cell populations in brain organoids, suggesting similarities with the developing human cortex in the second trimester of gestation [9,46]. Table 1 compares the different model systems in regard of their use in preclinical biomedical research. All these models have advantages and disadvantages. For example, a high number of drugs found to revert disease phenotypes in animal models fail to alleviate the same phenotypes in humans, suggesting that the translation between the mouse model and the human clinical symptoms is not wholly accurate[47].

Learning how viruses can affect human neurodevelopment

A Zika virus outbreak in Brazil was hypothesized to be the cause of congenital malformation in children[48]. The Zika virus is mosquito-borne, and thus, insects can act as a vectors that transmits the virus from one person to the next[49]. Once the pathogen is inside the host cell, a positive-sense mRNA is sent out to be translated and transcribed to generate copies of the virus, leading to a cascade effect within our bodies and quickly infecting our cells [50]. Due to the increasing number of infants being born in Brazil with microcephaly, scientists began exploring the idea of a link between the Zika virus and brain malformations[51]. Due to the species differences between embryonic formation of mouse and humans brains, a human model was imperative to suggest causality between the circulating Brazilian Zika virus and microcephaly [52,53].

Interestingly, the human brain organoid technology was sufficiently mature to provide researchers with a powerful experimental model. Brain organoids derived from human pluripotent stem cells (hPSCs) were generated and matured to a point when the cortical plate was evident. After the infection of the brain organoids with different strains of Zika, it became clear that the virus was targeting cortical progenitor cells, inducing apoptosis and autophagy, especially from deep-layer V/VI, which led to microcephaly [54]. These detailed observations of the target subcellular types affected by the Zika virus during neurodevelopment would be impractical using a mouse model due to the accelerated gestational time-frame compared with humans. The model not only taught scientists about the Zika-induced congenital disease but now can be used to determine the efficacy of future treatments to counteract the harmful impact of the virus[55].

Encephalitis is acute inflammation of the brain resulting from viral infection or an autoimmune attack on brain tissue[56]. A derivative of the disease known as Japanese Encephalitis (JE), which is caused by the Japanese Encephalitis virus (JEV), is characterized by production of proinflammatory cytokines and chemokines that synergistically trigger neuronal damage[57]. Only a handful of infected neuronal cells still express antiviral

interferon-stimulated genes, which have been identified in previous studies, indicating that the brain retains autonomous antiviral immunity in some cases [58]. Cerebral organoids were used to generate telencephalon organoids which were then infected with JEV. JEV seemed to preferentially infect astrocytes and neural progenitor cells located within the outer radial glial cells. JEV infection resulted in a decline in cell proliferation and a spike in apoptosis compared with the noninfected control organoids. Older organoids retained variable antiviral immunity [58]. The variable antiviral immunity in the late stages of development and infection of outer radial glial cells explains why JEV has a severe outcome in the young.

Brain organoid exposure to environmental toxins

Brain organoids can also be used to measure the impact of environmental exposure to toxic factors during prenatal neurodevelopment[59]. The effect of prenatal diseases on development of the fetal brain is difficult to study due to limited accessibility of human models and the physiology of animal models [60]. A common stimulant that causes long-term behavioral deficits in offspring is nicotine[61]. Nicotine has been known to trigger various neuronal disabilities in the fetal brain during pregnancy[62]. Brain organoids were used as an experimental model to determine the consequences of nicotine exposure in prenatal human neurodevelopmental. The nicotine-exposed organoids developed premature neuronal differentiation with enhanced expression of the ??-tubulin, typically found in newly generated immature postmitotic neurons [60]. Exposed brain organoids also expressed other neuronal markers, which disrupted brain regionalization and cortical development in the fore and hindbrain [60]. These results showed that nicotine exposure elicits impaired neurogenesis in the early fetal brain. The example highlighted here provides supportive evidence that brain organoids are a promising platform to model neurodevelopmental disorders induced by environmental factors. Future studies comparing traditional 2D neural cultures with brain organoids might stress the advantages of longitudinal studies with brain organoids in this particular field.

Modeling genetic neurological disorders with brain organoids

Sandhoff disease is a rare inherited disorder that progressively destroys nerve cells in the brain and spinal cord[63]. Sandhoff disease is part of a group of related genetic disorders that result from an absence of the enzyme beta-hexosaminidase, which catalyzes degradation of fatty acid derivatives known as gangliosides[64]. The disease is known to cause early childhood death due to deterioration of the brain. The exact relationship between ganglioside accumulation and neurodegenerative effects is not entirely understood. Patient iPSCs were derived, and a portion of the cells were corrected for the mutation in the β-hexosaminidase (β-*N*-acetyl-D-hexosaminidase, EC 3.2.1.52) β subunit (HEXB) allele via clustered regularly interspaced short palindromic repeats (CRISPR) genome editing, creating isogenic controls for downstream experiments[65]. Both iPSC types were then coaxed to generate cerebral organoids. The HEXB mutant-derived iPSCs accumulated G(M2) ganglioside and showed increased levels of cellular proliferation compared with the control cells. The HEXB mutant-derived organoids exhibited altered development, suggesting that G(M2) gangliosides experience neuronal differentiation early in development.

Three-prime repair exonuclease 1 (TREX1) is an antiviral enzyme that cleaves nucleic acids in the cytosol, preventing the interferon-associated inflammatory response[66]. When TREX1 is nonfunctional, autoimmune diseases, such as Aicardi-Goutieres syndrome (AGS) and lupus, can arise[67]. To model AGS, brain organoids from patients and CRISPR-edited iPSCs were generated. An abundance of Long Interspersed Nuclear Elements-1 (L1) retrotransposon extrachromosomal DNA was noted in TREX1 -deficient neural cells, especially in astrocytes [68]. The accumulation of L1 retrotransposon nucleic acids triggered an inflammatory response by activating the interferon pathway.

Moreover, TREX1 -deficient neurons were more prone to apoptosis, which reduced the overall size of the organoids. A reverse transcriptase inhibitor was used to block L1 retrotransposition in AGS brain organoids, preventing neurotoxicity[68]. Thus, this is one of the few examples in which brain organoids were used to dissect a molecular mechanism underlying cellular toxicity and to propose a novel clinical treatment.

Organoids as an oncology model

Organoids can also provide a platform in which brain tumor invasion can be studied *in vitro* [69]. Glioblastoma multiforme (GBM) is a form of brain cancer with a high demand for clinical attention[70]. GBM cells invade the parenchyma, which makes surgical removal nearly impossible[71]. Recently, cerebral organoids were used to model GBM invasion by creating a hybrid organoid with an invasive tumor phenotype that differed from the regular growth pattern of cerebral organoids [71]. By recapitulating GBM cell invasion in a cerebral organoid, anti-GBM therapies to block tumor invasion could be tested.

Conclusion and perspectives

Development of brain organoids from human pluripotent stem cells (hPSCs) is a recently established and exciting technique that can fill a gap in the current understanding of the early stages of prenatal human neurodevelopment. Because many conditions, such autism spectrum disorders (ASD), have embryonic origins, the impact of this tool cannot be underestimated. Consistently, the number of scientific insights gained from the use of brain organoids increases every year. The examples highlighted here (Figure 1) are only a snapshot of a fast-evolving field. Nonetheless, there are currently several limitations, both practical and theoretical, such as a lack of essential patterning cues for full cellular maturation, that will need to be addressed for proper maturation of this field.

Currently, the size of brain organoid models is limited due to the lack of a vascularization system[72]. Without blood vessels, proper nutrition cannot be given to all the cells, especially internal neural progenitor cells. Cerebral organoids have cell types similar to those found in the human brain, but the cells are likely not organized in the same way[73]. A real-life example of this would be to imagine the brain organoid as a disassembled bicycle, and even though all the parts are there, the bike will not work because it has not been put together correctly. These are just some of the technical issues related to the current brain organoid protocols. From a theoretical perspective, the scientific community needs to be aware of potential ethical concerns related to the use of this tool[74,75]. While most of the studies on

brain organoids to date used them for structural readouts (size, cell migration, gene expression, and cortical lamination), the formation of sophisticated neural circuits and even nested oscillatory waves is on the horizon[76]. Thus, healthy discussions about the implications of the use of this technique will need to occur at different levels of the society.

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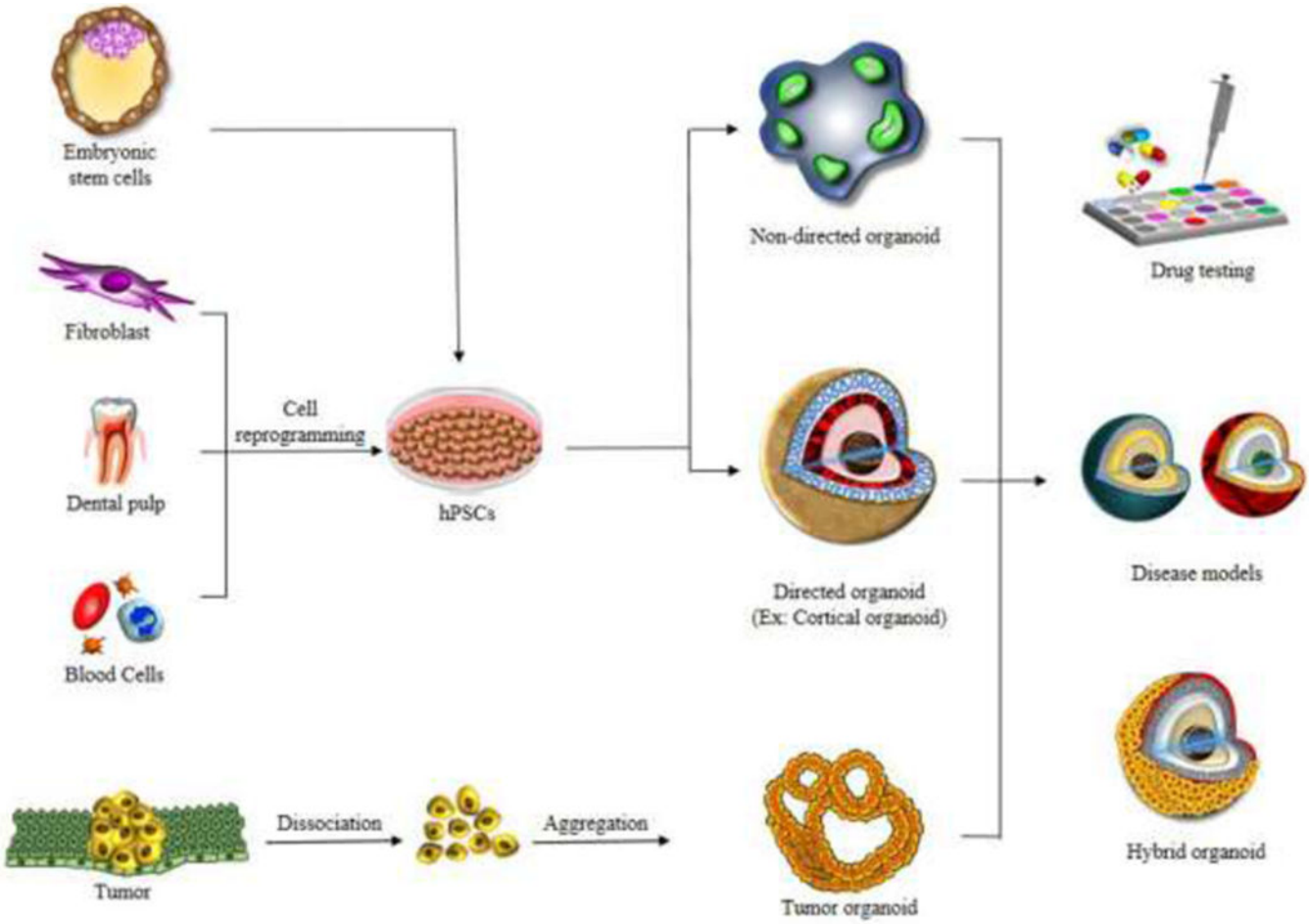

Figure 1: Potential applications of brain organoid models.

Brain organoids can be generated from human pluripotent stem cells (hPSCs), either embryonic stem cells or induced pluripotent stem cells reprogrammed from several types of primary somatic cells. During differentiation, brain organoids can be patterned to a specific brain region, such as the cortex. Nondirected or nonpatterned brain organoids would have different brain regions represented in a single unit. Organoids can also be generated from brain tumors. Brain organoids can be used to study the impact of drugs for specific disorders or toxicology. Comparing brain organoids derived from control and affected groups allows the researchers to perform disease modeling in a dish, revealing fundamental mechanisms of the underlying pathology. Finally, chimeric brain organoids can be used to create hybrid structures to understand the interplay between different cell types, such as during tumor progression.

Table 1:

A panoramic comparison of preclinical models of disease.

	Neuronal monolayer	Brain organoids	Animal models
System complexity	Low	Medium	High
Translational potential	Medium	TBD	Low
High throughput	High	Medium	Low
Cost	Low	Medium	High