Review on the use of 3D cerebral organoids and the current technical progress in their development

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Introduction

An organoid is an artificial mass of cells that operate like an organ. Neural organoids are organoids that model the central nervous system (CNS) and are also known as cerebral organoids. Research into these cerebral organoids is still in its infancy. With less than a decade of history being utilized, however; cerebral organoids are already becoming important for studying the progression of neurological diseases and even developing methods to treat them. This includes a wide range of neurodegenerative disorders like microcephaly, schizophrenia, ZIKA, autism, and Alzheimer's disease to name a few [8, 11, 14, 15, 16, 17]. The basis for this technology lies in the development of human pluripotent stem cells (PSC). PSCs. The modification of these PSCs leads to a variety of neural progenitor cells. From neural progenitor cells, monolayer or neural tubes can be studied in what is known as 2D culture systems. They allow for the relatively easy study of neural systems and very early embryonic modeling, as well as high-throughput screenings for a wide range of genomic testing. These 2D models however have their limitations when studying the longer-term development of diseases [2, 5, 7]. This is where 3D models (neural organoids) are necessary for modeling cell-to-cell and cell-to-extracellular matrix interactions [1, 9, 12]. These systems are currently however lacking important factors necessary for higher development like proper gas and nutrient exchange that are mediated by the vascular system. They also lack the complex glial cell integration necessary for cell guided migration, neuro-chemical control systems, and neurological immune responses. This review will focus on the current organoid design processes, the integration of vascular systems, and the development of glial cell differentiation.

Basics of an organoid

A neural organoid can be seen to have an organization in elaborate progenitor zones like the subventricular zone (SVZ) being split by an inner fiber layer [8]. This separates the SVZ and the outer subventricular zone (OSVZ) where intermediate progenitor and outer radial glia are found. This results in tissue that has clear cortical layer separation [9]. Further, large fluid-filled pockets can begin to form into ventricles and in some cases, retinal tissue forms [8]. Organoids also form distinct cerebellar areas like the forebrain, hindbrain, parietal, and occipital lobe. The cells of the organoids also begin differentiating into the hippocampus and ventral forebrain but more complete structural formation isn't normally attained [8, 9, 17]. Protocols exist for making 3D organoids form human PSCs and proceeding to the production of an embryoid body [8, 9]. These embryoid bodies are then maintained in a suspension fluid until they develop into neural rosettes, where they are then placed in a Matrigel droplet [8,9]. This Matrigel droplet acts like a mesoectoderm, allowing the neural progenitors to have a scaffold [1]. The progenitor cells, that have now matured into embryoid bodies, can use this scaffold to further develop the complex processes of neuronal migration, maintenance of apical-basal polarity, and neuronal stem cell differentiation [8,9]. These Matrigel droplets are then placed in a spinning bioreactor to better facilitate the perfusion of nutrients and gases in the organoids. Due to this perfusion organoids

have been shown to survive for months [18]. This basic model depends on the intrinsic signaling of the hPSCs. Some examples of this would be in the expression of FOXG1, EMX1, and AUSTS2 [8]. These are all molecules that signal the formation of specific regions and these molecules help decide cell phenotype expression. There is also glial cell signaling and metabolic modulation to consider, which aids the development of the organoid [10, 13]. This development continues until the organoid meets with growth inhibition due to the inability of nutrients to perfuse into deeper cell layers. This inability to perfuse nutrients to the core of the organoid results in necrosis of cells therein. However, further research has shown that this protocol can be modified with the addition of further extrinsic growth factors. These can modify the organoid to model specific regions of the brain such as the midbrain, hippocampus, cerebellum, and hypothalamus. These factors include LDN-193189 for the midbrain and A83-01 for the forebrain [7, 17]. In the focus of one article, forebrain organoid formation involved the use of a stem cell medium with Dorsomorphine and A83-01 added, but without FGF-2, for the first 4 days [17]. On days 5-6 this medium was changed to include DMEM:F12, 1X N2 Supplement, Heparin, Penicillin/Streptomycin, Non-essential Amino Acids, Glutamax, WNT-3A, CHIR99021, and SB-431542 and was used to maintain organoids for more than 100 days [17]. Other than developing organoids that model specific brain regions, neural organoid research has focused on modeling degenerative diseases with the goals of learning about disease etiology and developing potential treatments for them. This research has focused on various diseases including, but not limited to Alzheimer's, ZIKA, microcephaly, and the treatment of glioblastoma [8, 11, 14, 16, 17] . In the case of the ZIKA virus, organoids were used to provide evidence for the assertion that the virus had an effect on neuronal development. Some organoids were grown normally to represent the control group and two other groups were infected with 2 different strains of the ZIKA virus. What was determined was that there were specific developmental deficits expressed in the ventricular zone of the infected groups. These deficits were the statistically significant decreases in cortical layer thickness and cell proliferation count in the ZIKA infected organoids when compared to the non-infected organoids [17]. In the use of studying cancer, cultures of a patient's glioblastoma were implanted into neural organoids. In vitro tests then were performed to determine the efficacy of temozolomide and doxorubicin as treatments for the glioblastoma while not harming the neuronal cells [16]. These tests were also conducted when the organoids were surgically implanted in mice. What was determined was that doxorubicin was more effective in reducing the size of the glioblastoma, while also preserving the neural tissues. Something that was mentioned in the discussion was the inability to assess how well the perfusion of the organoids impacted the interaction between the glioblastoma and the drug interaction [16]. The lack of vasculature was said to likely change the behavior of glioblastomas, as they follow vasculature, using it to metastasize. Also, while the drugs used in the study were able to perfuse to the center of the organoid tissue, this process took between 6-12 hours and, had a vascular system been present to aid in the rapid delivery of the drugs, the interaction may have been different between the drugs and the cells.

Vascular integration

Vascularization is an area that is burgeoning and exciting in organoid technology. A few studies have been done by integrating and differentiating epidermal cells (derived from human embryotic cell-lines), which begin to make vascular epithelial tissue that innervated the organoids, followed by the implantation of the organoids in a lesioned mouse cortex. The lesion was performed by excising a small 1X1 mm square of neural tissue from the mice's brains. This has been used as a technique to observe how well-lesioned areas can adapt to implanted neuronal tissue [3]. Organoids have also been implanted into mice without any vascular system preparation, observing how the host's vascular system penetrated the organoid. The degree of integration of the organoids with the host system was reduced and there was a higher rate of rejection when compared to implanted organoids that had a pre-existing vascular structure [7]. It should also be

noted that the mice's microglia infiltrated the organoids, potentially altering the organoid's function. Recently, the incorporation of mesodermal progenitor cells in the Matrigel demonstrated the integration of an *in vitro* model with a more extensive vascular system. More extensive in terms of various cell types beyond endothelial cells, among which were pericytes, smooth muscle cells, a basal lamina, and blood vessel valves. An interesting finding was also that when the organoids were exposed to anoxic environments, around 2% oxygenation between days 1-7 of incubation, there was significantly more vasculature that was identified to have invaded the core of the organoid [19]. These vessels were tagged with CD31 fluorescent protein on αSMA and low-density lipoprotein (which occurs on smooth muscles and endothelial cells respectively) to identify the vasculature structure [19].

Glia

Glial cell formation appears to be inherently occurring in cerebral organoids [13, 18]. Using organoid models can offer insight into how glial cells aid in the development of the cortex [18]. In the past, it had been difficult to identify the degree of glial development in organoids[12,18]. This difficulty is due to the limitation in the development of organoids currently, which is akin to around the first trimester of embryonic development, as glial cells do not mature and differentiate fully. This does allow one the unique opportunity to culture astrocytes that have not yet acquired phenotypic specificity though, which can help study how astrocytes generally function [4, 5]. Difficulty had been previously expressed with proliferating and maturation of microglia in organoids due to dual-SMAD inhibitors. These dual-SMAD inhibitors are used as precursors to proliferating neural progenitor cells, but new research indicates that there is evidence of microglial maturation innately in organoids despite this obstacle [13]. Proper microglial development has also been an issue, as some microglia develop from non-ectodermal cell lineages outside the central nervous system, which has yet to be introduced into organoids [13]. This is important, as glia, in general, are vital for proper neurogenesis, synaptogenesis, metabolic modulation, and differentiation of neurons [13, 18]. Particularly, embryonic microglia in humans have a profound effect on maturation and development of neural tissue, especially in the subventricular zone [10]. Glial cells also have been shown to be important in studies focusing on mechanisms that affect tri-partied synapses in diseases such as Alzheimer's [14]. In a study conducted by Park. et al., it was important to see how glia were affected by Alzheimer's disease, as these reactions can further the processes that result in cell death. These factors included: neuroinflammatory activity that deleteriously affects neurons and astrocytes, neurotoxic activities that result in axonal cleavage, and nitrous oxide release that damages both neurons and astrocytes [14]. It is important to consider glial interactions in development, as proper development is not possible without them, and diseases that target glial cells, like multiple sclerosis, need proper glia cell interface to be accurately modeled. With an accurate model, one can determine mechanisms that lead to impaired function and ultimately develop methods to prevent or fix the problem.

Conclusion

Cerebral organoid development is still in its initial stages. There have already been strides toward more complex and well-developed models with further research in identifying more effective methods for introducing blood vessels and proper glial cell maturation and function, however; an artificial system that can induce blood flow and further glial interaction with the mesoderm, are necessary for further development in organoids. This blood flow, or the flow of the nutrient-rich incubation fluid (which contains glucose, 21 amino acids, fats, and oxygen among other nutrients), through the organoid is possible with the aid of a host animal but this can introduce a variety of variables that may cause complications. Further maturation of the neural organoid is also halted. A method that could overcome this involves integrating a peristaltic pump (in the same manner demonstrated in kidney organoids [6]) with the developing

mesodermal derived vascular tissue in a neural organoid. This would help circulate the incubation fluid in neural organoids and could lead to increases in maturation of the neural tissues. These steps are also necessary for better organoid transplant success as the tissues would undergo more changes that are like those developed *in vivo* [19]. These changes refer to the refining of the vascular system, due to the cellular responses to fluid movement and pressure in the vessels [19]. These organoids would be better developed and as previous research shows; this would aid in transplantation success in hosts [3, 6, 7, 18, 19]. This could aid individuals who have traumatic brain injuries in receiving organoids developed to replace the specific lost tissue. The lack of glia driven research is also something that needs to change in neural organoid research. While glial research has been receiving more attention in recent years, as recognition of their influence on neuronal function and development has become more and more evident, more glial focused research is needed to develop better neural organoids. This should be focused on embryonic microglia, specifically the non-ectodermal cell lineage glia that migrate into the brain before the closure of the blood-brain barrier. Current models lack these cells and organoid development could be improved with their integration. There is also a large lack of research in implementing a blood-brain barrier which requires astrocytes to interact with meningeal tissue. By adding meningeal precursor cells to the matrigel, further research could be done on the interaction of astrocytes and the meninges in the formation of the blood-brain barrier. The interaction between the meninges and astrocytes may have a further developmental role in shaping neural proliferation, which has yet to be researched in organoid models. By better modeling the human brain, neural organoids could become invaluable assets in understanding the biology of human brain development. This could lead to growing tissue replacement therapies, better treatments for debilitating neurological diseases, and may even help with improving neuro-computational interfaces.

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