Approaches in vivo and in vitro for solving the vascularization issue of brain organoids

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Abstract. Organoids are three-dimensional tissue cultures generated using human pluripotent stem cells. It shows great potential in modeling human disorders, organogenesis, and developmental disorders. However, they have limitations in replicating the complexity of the human brain and lack a complete vasculature to support long-term cultivation and endogenous microenvironment simulation. Researchers are exploring strategies to promote vascularization in brain organoids, including in vitro co-culture methods and xenotransplantation into highly vascularized regions of animal hosts. The presence of a physiologically perfused vasculature in organoid models can prevent tissue necrosis, provide essential nutrients, and enable accurate modeling of interactions with non-neuronal cell types. In vivo, xenotransplantation shows advantages over in vitro approaches, such as active blood flow, and demonstrates potential benefits for aiding recovery from stroke by repairing damaged tissue structures and improving sensory-motor deficits.

Keywords: Organoids, Vascularization, Xenotransplantation, Active blood flow, Stroke recovery.

1. Introduction

As a result of the rapid advance of technique, human pluripotent stem cells (hPSCs) become an invaluable tool in many aspects [1] including modeling human disorders organogenesis, and developmental disorders. hPSCs have the ability to differentiate into all types of cells or tissue that exist in the human body under certain conditions. This ability makes it capable of generating three-dimensional (3D) tissue cultures that mimic organs and contain various cell types termed organoids.

Organoids are valuable as disease models, they also show potential as an alternative source for tissue repair organ transplantation, a study shows transplanting brain organoids can rebuild damaged tissue and repair neural function deficits in stroke mice [2]. However, organoids have limitations, such as an inability to fully replicate the human brain and a lack of vasculature, which can lead to necrosis in the center of the organoid and affect its development. Notable efforts have been made to overcome this limitation. A classic example of such an attempt is to assemble the cortical brain organoid with vascular spheroid to achieve vascularization [3]. Other methods include in vitro co-culture methods involving vascular cells or integrating with blood vessel organoids, optimizing culture conditions to stimulate vascular cell growth, employing genome-editing techniques to induce endothelial cells in unconventional locations, and developing microfluidic "organoid-on-a-chip" systems.

In addition to the widespread issues related to vascularization, brain organoids have other limitations including the inability to fully model the interaction between intrinsic genetic regulatory mechanisms and extrinsic factors, such as signaling molecules and cell-cell interactions, which are essential for the formation of cortical layers [4-6]. Application of human brain organoids—Opportunities and Challenges in Modeling Human Brain Development and Neurodevelopmental Diseases] Lacking immune cells like microglia.

Brain organoids may be missing certain cell subtypes, and lack some brain structures. Additionally, they might not fully replicate the intricate circuitry required to model complex brain diseases or normal cognition. In addition, they remain immature, resembling fetal stages of the human brain, which can impact their ability to accurately represent adult brain tissue. The absence of certain cell types found in the human brain can further limit their effectiveness in accurately modeling brain

function and disease. For these reasons, the reliability of brain organoids can be doubtable for some applications and may fail to represent the human brain in aspects of the spatial organization of different cell types and brain regions [7].

This review provides an overview of the techniques used to generate brain organoids and explores their unique potential. It also addresses common challenges encountered in brain organoid experiments and applications, discusses methods developed to overcome these limitations, and analyzes potential avenues for the development of brain organoid technology.

2. Current technique for generating brain organoid

Organoid generation utilizes its own self-organization capability granted by PSCs. In general, there are two main methodologies for creating brain organoids: unguided and guided methods. Unguided methods rely entirely on the natural development and inherent differentiation abilities within hPSC aggregates, while guided organoid methods involve the addition of external patterning factors to direct hPSCs towards specific lineages. The number and combination of external factors used in differentiation protocols can vary, and the choice between unguided and guided approaches is often considered a balance between diversity and consistency [8].

2.1. Unguided brain organoids

The concept of the unguided brain organoid, also known as the cerebral organoid, draws inspiration from techniques used to create gastrointestinal organoids [9]. In a method developed by the Knoblich group, embryoid bodies (EBs) derived from hPSC aggregates are enclosed in an extracellular matrix (ECM), such as Matrigel, and then cultured in spinning bioreactors to encourage tissue expansion and neural differentiation [10]. This approach allows for minimal external intervention and enables cerebral organoids produced through this method to exhibit significant self-organization. They sometimes develop highly elongated neuroepithelial structures and display diverse cell lineage identities including forebrain, midbrain, hindbrain, retina, choroid plexus, and exoderm [11]. Through large-scale single-cell transcriptomic profiling, it has been revealed that cerebral organoids contain neural progenitors, excitatory neurons, inhibitory neurons, astrocytes, and oligodendrocyte precursor cells (OPCs) found in the CNS. Additionally, they may include photosensitive cells found in the retina [12]. However, due to the stochastic nature of hPSC spontaneous differentiation unpredictable proportions and heterogeneous arrangement of each lineage across batches of differentiated organoids can be observed. This diversity offers a unique opportunity to model interactions between different brain regions but presents challenges for systematic and quantitative studies due to high variability and heterogeneity.

2.2. Guided brain organoids

The Sasai group pioneered the development of 3D differentiation protocols for brain organoids using guided methods [13-18]. which involve the use of small molecules and growth factors to instruct human pluripotent stem cells (hPSCs) to form cells and tissues representative of specific brain regions [19-21, 17, 22]. These directed organoid cultures aim to generate consistent mixtures of cell types with minimal variation across batches and cell lines [23]. However, excessive use of external factors may interfere with hPSC self-organization and cell-cell interactions, leading to less well-defined cytoarchitecture in the resulting organoids. To address this issue, guided organoid differentiation protocols can be tailored to minimize the use of external patterning factors after successful initial stage patterning, allowing subsequent differentiation to follow intrinsic programs similar to those operating in vivo after neural patterning. Additionally, synthetic biomaterials such as microfilaments can be engineered to physically guide the formation of brain organoids, resulting in more consistent formation of enlarged ventricular structures and neuroepithelium [24-25]. Spinning bioreactors and miniaturized multi-well spinning bioreactors have been developed to improve the efficiency of organoid culture, enabling the generation of brain region-specific organoids that mimic

specific developmental features, offering unique advantages for understanding human cortical development and disorders [9, 10, 25].

2.3. Fused organoid technologies

While the methods for generating cerebral organoids can produce tissues resembling different brain regions, their proportion and spatial arrangement are highly varied and difficult to predict. To enhance the modeling of interactions between brain regions, several research groups have simultaneously developed new approaches. They first differentiate hPSCs into specific organoids representing different brain regions separately and then merge them to create organoids with multiple distinct region identities in a controlled manner [26-28]. For instance, fused dorsal and ventral forebrain organoids have been demonstrated to form an "assembloid" with two distinctive but interfacing domains [27]. In these structures, interneurons generated from the ventral domain preferentially migrate towards the dorsal domain, mimicking the tangential migration of interneurons from the subpallium to the cerebral cortex in vivo [29]. Furthermore, electrophysiological characterization of forebrain assemblies revealed that migrating interneurons synaptically connect with local excitatory neurons to form microcircuits [27]. Overall, recent advancements in brain organoid methodologies have expanded our tools for modeling human development and disorders. The choice between guided and unguided methodologies will depend on the specific focus of the investigation. For example, unguided organoids are suitable for exploring cell-type diversity during whole-brain development while brain region-specific organoids better replicate brain cytoarchitecture with less heterogeneity. Assemblies allow for investigating interactions between specific brain regions with more consistent molecular and functional characterization." [8].

2.4. Unique potential of brain organoids

Besides these attempts, there are multiple studies that revealed the unique potential of organoid transplantation, including the potential to aid recovery from stroke by repairing damaged tissue structures and improving sensory-motor deficits.

Transplanting brain organoids into mice may address issues related to nutrient delivery and necrosis. A study in 2018 indicates that brain organoids transplanted into mice exhibit a progressive pattern of neuronal phase-specific differentiation, develop functional vascular systems, demonstrate unprecedented axon growth, and consequently generate mature and functional human brain tissue in vivo that responds to physiological stimuli induced by anesthesia, additionally, functional synaptic connections between transplanted organoids and the host brain was detected with the help of optogenetics [30]. Studies also test the potential of the brain organoid to repair damage caused by stroke [31].

Stroke is a highly debilitating disease and one of the leading causes of mortality and disability worldwide. While cell therapy has been explored as a treatment for stroke, it presents challenges in repairing the tissue structures within the infarct core. A study demonstrated transplantation of human brain organoids successfully restored the tissue architecture within the infarcted area and substantially ameliorated sensory-motor deficits in stroke mice. This highlights the potential of organoid transplantation for stroke recovery.

Several months after transplantation, the study observed robust survival and differentiation of transplanted organoids within the infarct core. These organoids differentiated into target neurons, facilitated the repair of infarcted tissue, projected axons to distant brain targets, and integrated into host neural circuits. Consequently, this integration led to the elimination of sensory-motor deficits in stroke mice. Notably, researchers in this study found that single-cell transplantation derived from the organoids failed to achieve similar restoration of infarcted tissue, this suggests the unique properties of organoids make them perform better in tissue repair [31].

3. Challenges encountered in brain organoid experiments and applications

3.1. Approaches for solving vasculature issue

Organoid models can benefit significantly from the inclusion of an active and physiologically perfused vasculature. One advantage is the prevention of necrosis and cell death, which can greatly compromise the integrity and long-term culture of organoid models. Additionally, this vascular system provides essential nutrients that are currently lacking in traditional in vitro paradigms. The presence of a vascular system within brain organoids holds great value, not only for nutrient supply but also for potentially enabling accurate modeling of invasion/extravasation and interaction with various non-neuronal cell types [32].

To address these challenges, researchers are actively investigating several strategies to promote the vascularization of brain organoids. These include in vitro co-culture methods involving vascular cells [33-34] or integrating with blood vessel organoids [35-36], optimizing culture conditions to stimulate vascular cell growth [37], employing genome-editing techniques to induce endothelial cells [38] in unconventional locations, and developing microfluidic "organoid-on-a-chip" systems [39] Organoid models with functioning and physiologically perfused vasculature offer several advantages. One key benefit is the prevention of necrosis and cell death, which can significantly undermine the stability and longevity of organoid cultures. Additionally, the vascular system provides essential nutrients that are typically lacking in current in vitro setups. The incorporation of a vascular network within brain organoids is particularly valuable not only for nutrient supply but also for accurately modeling interactions with various non-neuronal cell types, potentially facilitating realistic representations of invasion and interaction processes [32].

A different research project investigated a specific approach to xenotransplantation, in which human brain organoids were implanted into highly vascularized brain regions of an animal host to promote vascularization of the developing organoid tissue. In an investigation, Mansour et al. [30] transplanted cerebral organoids derived from human ESCs into the retro splenial cortex of immunodeficient mice and observed significant infiltration by blood vessels and the establishment of functional circulation within the organoid grafts.

The initial investigations aimed at stimulating the vascularization of brain organoids in vivo utilized a technique for intracerebral transplantation originally designed for transferring fetal or adult tissue into the rodent brain (18-20). This method facilitated adequate infiltration of the transplanted brain organoids by the host's vasculature system, which became evident after 7 to 10 days in vivo; extensive vascularization was observed after 14 days post-engraftment [30]. Significantly, through two-photon microscopy, it was demonstrated that the blood vessels infiltrating the organoids exhibited robust blood flow. The survival of organoid transplants relied heavily on successful vascularization [30]; minimal cell death and reduced cellular stress were noted in brain organoids in vivo [30], [40]. Consistent with this discovery, a recent study indicated that activated cellular stress pathways in cortical organoids in vitro could hinder proper cellular subtype specification and neural progenitor maturation [41]. Upon transplantation into immunocompromised mouse pups' cortices, dissociated organoids displayed heightened levels of cell subtype specification in both outer radial glia and newborn neurons. Although only dissociated organoid cells were analyzed in vivo by the group [41], their findings suggest that transplantation partially alleviated cell stress and subtype defects observed in vitro. Nonetheless, a more comprehensive analysis is required to compare dissociated and whole organoid grafts in vivo. It is noteworthy to mention another study reporting improved survival and increased vascularization in cerebral organoid grafts compared to transplants using dissociated neural progenitor cells [42]. Engrafted organoids exhibited an augmented number of neurons and underwent a progressive transition from progenitor-dominated, rosette-rich tissue to a more mature composition predominantly consisting of neurons and astrocytes within living organisms [30]. Further investigation is necessary to evaluate intrinsic and environmental factors enabling sufficient vascularization of brain organoids in vivo. Alternatively, introducing human vasculature before transplantation seems to be a promising strategy for potentially replacing host

vasculature with human cells. While not extensively explored, artificially vascularized brain organoids displayed some degree of functional perfusion by animal hosts upon transplantation [33], [38].

3.2. Comparing in vivo and in vitro approaches to solve the limitations of brain organoids

In the aspects of vascularization, in vivo, brain organoid transplantation shows some advantages compared to in vitro brain organoid experiments, including the lack of active blood flow which some in vitro methods. A study achieved vascularized brain organoids via the fusion of brain organoids and vessel organoids. The vascularized brain organoids in this study exhibited an increased pool of neural progenitors and reduced apoptosis. The grafted BOrs with invading host blood vessels also showed a similar reduction in apoptotic cells [30], [34]. However active blood flow that is shown in organoids that have been grafted in vivo allows integration of vessels between organoids and the hosts as that done in other studies [33], [38], was not detected in this study.

4. Conclusion

Organoids are valuable as disease models, but they have limitations, such as an inability to fully replicate the human brain and a lack of vasculature, unable to fully model the interaction between intrinsic genetic programs and extrinsic factors, such as signaling molecules and cell-cell interactions, which are essential for the formation of cortical layers, missing certain cell subtypes, lack some brain regions. Researchers are exploring various strategies for promoting vascularization in brain organoids, including in vitro co-culture methods and xenotransplantation into highly vascularized brain regions of animal hosts. Organoid models benefit from a physiologically perfused vasculature can prevent necrosis, provide essential nutrients, and enable accurate modeling of interactions with non-neuronal cell types. In vivo, xenotransplantation for overcoming the vasculature issue shows advantages in some aspects compared to in vitro approaches, including active blood flow. In vivo, xenotransplantation also shows potential in other aspects, such as the potential to aid recovery from stroke by repairing damaged tissue structures and improving sensory-motor deficits.

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