Progress in the construction and application of neural organoids

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Abstract. With the establishment of three-dimensional (3D) cell culture methods, embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), or tissue-resident stem cells/progenitor cells can be cultured in vitro to produce structures similar to organs, called "organoids".Compared with the traditional model, neural organoids have unique advantages, such as being able to perform high-throughput drug screening, organ development simulation in vitro, and predict the individual response to drugs more accurately. Therefore, combining the advantages of organoid models with traditional models will open up new avenues for human biology. Based on these advantages, neural organoids have become an in vitro model that has attracted much attention in recent years. This review summarized the current research progress of neural organoid models, and discuss the challenges and development prospects of neural organoids.

Keywords: Neural organoids; neurodevelopmental disorders; neurodegenerative disease; toxicity testing; drug screening.

1. Introduction

Organoid culture is a new approach to the study of human development and human disease. They are similar to primitive human tissues, containing the correct cell types and performing certain tissue functions. Because they faithfully encapsulate aspects of tissue composition, structure, and function in vitro, they open up tremendous possibilities for identifying new therapeutic strategies in personalized human disease models [1].

Depending on the cell source, organoids are classified as adult stem cells (ASCs), pluripotent stem cells (PSCs) or patient-derived organoids (PODs), while PSCs are divided into embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSC) [2].Organoids from different sources have different characteristics and uses, for example, neuroectoderm such as optic cup and brain[3] organoids are only derived from PSCs, so PSC organoids are mainly used for the study of psychogenetic diseases and reproductive biology, while ASCs are derived from precursor cells with regenerative ability in tissues, which are mainly used in the study of adult tissue biology, tissue regeneration, and precision medicine.

Compared with traditional models, organoids have the following characteristics: (1) self-assembly to form a 3D structure, under the regulation of exogenous signals, multi-directional differentiation self-constructs into tissues or organs similar to their origins, simulating the incubation process of organs in vivo [4]; (2) they have some specific functions of organs, such as the respiratory function of the lungs, nutrient absorption of the intestines, and filtration function of the kidneys, etc. [4]; (3) they can be widely derived and can amplify a large number of genomes to maintain the stability of the genome [5], they can be gene-edited and high-throughput screening. According to the characteristics of organoid models, they can be applied in a complementary way to traditional models, rather than being replaced, which will open up new ways for human biological research.

2. Construction methods of neural organoids

2.1. Methods for building neural organoids

The traditional method to construct neural organoids is using primary stem cells. In recent years, embryo bodies (EBs) produced from pluripotent stem cells have been widely used due to their self-

propagate and multipotent differentiation ability. EB is the precursor of organoids, which can develop several kinds of disease models. Commercial ultra-low attachment (ULA) V-bottom well plates are frequently used to generate EBs. Because these plates are relatively expensive, a cost-effective and low-labor-intensive method has subsequently emerged to generate EBs: rinse standard untreated tissue culture plates with antiadhesion solution prior to cell seeding, reduce surface attachment by applying antiadhesion solutions, and centrifuge to enhance cell aggregation, creating homogeneous EBs in standard V- and U-shaped bottom plates. EB manufactured using centrifugation has less variability in final size than non-centrifugal method, and centrifugation also increases EB yield [6]. It is expected that this easy-to-implement approach will greatly facilitate the generation of EB, further support the organoid research.

Another method for preparing organoids besides using stem cells is cell reprogramming. For example, fibroblasts are reprogrammed through a combination of specific transcription factors (TFs) to transform into induced autonomic ganglion (iAG) organoids. The iAG organoid exhibit the molecular characteristics, subtype diversity, and electrophysiological characteristics of autonomic neurons. In addition, they can be integrated into the superior cervical ganglion after transplantation and innervate and control the beating rate of co-cultured ventricular myocytes. Therefore, iAG organoids may provide valuable tools for studying the pathogenesis of autonomic nervous system diseases and screening drugs [7].

2.2. Construction of complex neural organoids

Neural organoids are derived from ectodermal neuroepithelial cells that lack blood vessels, lymphatic vessels and immune cells. Although the endothelial layer, medial smooth muscle compartment, and outer membrane connective tissue have been shown to contain nonvascular and vascular progenitor cells that contribute to the formation of new blood vessels, incorporation of endothelial cells into neural organoids is not sufficient to produce functional blood vessels. Notably, organoids with a multilayer vascular functional network can derived from mesodermal progenitor cells [8]. Co-culture of mesenchymal and neural organoids has recently been shown to produce partially vascularized neural organoids [8]. Moreover, the use of engineering methods such as biological manufacturing or 3D printing is also an effective way to produce vascularization of organoids [9].

In addition to blood vessels, the immunization of organoids is also important. Macrophages are resident in all organs and tissues of the body, which are potentially involved in developmental processes and morphogenesis. In addition, macrophages may become important players for disease modeling of organoids. Brain-specific macrophages are called microglia. Several groups have given rise to microglia-sufficient neural organoids by coculture. They exhibited a microglia-like phenotype with microglia-specific gene expression profiles, and are capable of phagocytosis [10]. By adding stimulating cytokines, the number of macrophages can be further increased and more precisely controlled. Previous studies have shown that microglia can be differentiated from induced pluripotent stem cells (iPSCs) and co-cultured with different types of neural organoids. The incorporation of microglia into brain organoids can also be achieved by transplanting organoids into mouse brains [3].

2.3. Engineering neural organoids

Engineering strategies, means the use of some special materials and equipment to build neural organoids. Three-dimensional (3D) bioprinting is an emerging method for constructing neural organoids that can overcome the limitations of self-organization. It requires the development of optimal hydrogels. Gelatin is a valuable adjunct to 3D bioprinting due to its viscosity and biocompatibility. In addition, collagen, fibrin, hyaluronic acid, and laminin can provide biological support for adhesion, movement, differentiation, or synapses, and optimize the 3D culture of nerve cells. Therefore, optimizing specialized hydrogels to facilitate stem cell differentiation and increasing resolution will further improve the quality of bioprinted brain models[9].

Another method is to embed magnetic nanoparticles locally into organoids. The results suggest that the morphology, growth, and patterning of whole-tissue organoids can be guided by stimulating internal local tissues. These local mechanical forces are achieved by embedding local magnetic tissue, which can generate rapid or slow-changing forces, with rapid short-term stimulation deforming local tissues and long-term stimulation triggering cytoskeletal rearrangement, differential growth at tissue scale, and remodeling of cellular structure. Thus, by aggregating magnetically labeled human pluripotent stem cells in a certain order and then being driven by a magnetic field, local magnetic clusters can be generated within the organoids. These clusters exert local mechanical forces on surrounding tissues in response to the applied global magnetic field. Studies have shown that precise, spatially defined stimuli provide short-term mechanical tissue perturbations as well as long-term cytoskeletal remodeling. Local magnetic stimulation induces asymmetric growth and proliferation, leading to increased capacity of human neural organoids. This approach allows local, controlled mechanical stimulation in multicellular constructs and is widely used to study the role of local mechanotransduction in developmental and disease model systems[11].

3. Application of neural organoids

3.1. Disease modeling

3.1.1. Neurodevelopmental disorders

Many neurodevelopmental disorders, including cortical malformations, are associated with features that are more difficult to model, including generalized neurodevelopmental delay, epilepsy, ADHD, and autism [12]. Seizures can be identified by altering neuronal eruption activity and local potential [13]. Thus, neural organoids using induced pluripotent stem cells from patients can successfully mimic Rett syndrome and schizophrenia.

Some features of autism have also been successfully modeled in neural organoids.[2] autism spectrum disorder (ASD) refers to a complex group of neurodevelopmental disorders caused by genetic or environmental perturbations during early development, which cause patients to have difficulty communicating with others, often engage in repetitive behaviors, and limit the patient's interests, affecting the patient's ability to function normally in different areas of life [14]. Diagnosis relies on identifying behavioral abnormalities, but these abnormalities may not appear until long after the disease is established, so the key sites of treatment cannot be characterized. In recent years, advances in stem cell technology have created opportunities to mimic ASD in the human environment through the use of pluripotent stem cells (hPSCs). The stem cells can be used to generate 2D cell models as well as 3D unguided region-specific neural organoids. These models produce very complex systems capable of spatially mimicking the developing brain to reproduce key sites throughout early development. When complemented by multiomics, genome editing, and electrophysiological analysis, they can be powerful tools for analyzing the neurobiological mechanisms behind this complex disease [15].

In addition, the use of induced pluripotent stem cell-derived ventral and dorsal forebrain organoids also mimicked Timothy syndrome. This is a genetic disorder caused by mutations in the L-type calcium CACNA1C channel associated with epilepsy and ASD. By assembling ventral and dorsal forebrain organoids into so-called "combinations," the integration of GABA interneurons with functional microcircuits during brain development and the migration of salts to the cerebral cortex can be modeled [16]. Saltatory migration is a periodic movement in which neurons guide an extension in one direction during the process, followed by transient swelling of somatic cells and nuclear translocation (nuclear movement) [17]. Repeating these steps yields a typical saltatory pattern of migrating neurons. When assembled with dorsal forebrain organoids, ventral forebrain organoids tagged with reporter genes of GABA interneurons can show disrupted salinization patterns that ultimately delay neuronal migration in live imaging, thus mimicking the disease [16].

Human-animal neural chimeras resulting from in vivo transplantation of iPSC-derived nerve cells and organoids into animal models also have huge potential uses in disease modeling [18]. An important study illustrates the use of neurochimeras to mimic neuropsychiatric disorders, particularly Down syndrome (DS) [14]. Chimeric rodents using human DS organoid xenograft can exhibit excessive GABAergic interneurons and impaired performance on memory tests [19]. In this context, the development of human-rodent chimeras will help to study the interaction between abnormal gene expression and human interneuron development in vivo, which will provide new insights into the pathogenesis of Down syndrome and the regulation of specific genetic targets [14].

The tuberous sclerosis (TSC) complex is an autosomal dominant systemic disorder characterized by epilepsy, ASD, mental retardation, and mental manifestations associated with mutations in the TSC1 and TSC2 genes [20]. TSC1 and TSC2 mutations result in increased target activation of the mammalian rapamycin (mTOR) complex, which responds to growth factors and nutrients involved in cell proliferation and metabolism. More recently, by introducing TSC2 and TSC9 mutations with CRISPR-cas1 editing in human embryonic stem cells (hESCs) and patient-derived induced pluripotent stem cells (iPSCs), cortical organoids can be generated to mimic tuberous sclerosis. Rapamycin in TSC organoids prevents mTOR overactivation in early TSC2 homozygous knockout cultures and rescues cell hypertrophy. In addition, removal of rapamycin from organoids treated early is associated with hypermTOR, suggesting that long-term use of mTOR for TSC may be required [21]. Thus, neural organoids are allowed to study the effects of currently available drugs at the multicellular scale and can faithfully recapitulate the cellular structural characteristics and genetic expression capacity of complex neuropsychiatric diseases such as TSC [14].

However, neurodevelopmental disorders are often comorbid, have complex and heterogeneous genetic causes, and are characterized by behavioral manifestations, which complicates research using neural organoid models. For example, disruption of mTOR signaling is associated with a range of neurodevelopmental disorders that often accompany epilepsy [12], and mTOR disease-related disorders can lead to layered structural disturbances, altered cell morphology, migration, and connectivity, and disruption of ion channel function, affecting physiological activity [22]. Relevant tissue and physiological features may not be easily modeled in organoids [12].

3.1.2. Neurodegenerative diseases

Neural organoids have also been successfully used to mimic neurodegenerative diseases[18]. Neurodegenerative disease is a disease caused by the loss of neurons or their myelin sheaths in the brain or spinal cord that worsens over time, leading to dysfunction. Neurodegenerative diseases can be divided into two groups according to phenotype: one type that affects memory, such as Alzheimer's disease[23]; another type affects movement, such as cerebral ischemia, Parkinson's disease[23].

Three-dimensional microfluidic platforms with neural organoids are becoming powerful tools in neurobiology because they can control and manipulate the brain's microenvironment in a precise way[24]. For example, reconstructing a three-dimensional (3D) human culture system for understanding neurodegeneration and neuroinflammation in Alzheimer's disease (AD). They created an engineered model system in the AD environment containing neurons, astrocytes and microglia, enabling understanding of human microglial aggregation, neuroinflammatory responses, and damage to neurons or astrocytes. This developed 3D human AD triculture model system demonstrates two well-established progression pathways in Alzheimer's disease: Aβ aggregation and hyperphosphorylation of tau, and elevated expression of chemokines and cytokines. The model provides an interesting platform for exploring mechanistic insights into AD and screening new therapeutic clues[25].

Cerebral ischaemia causes inadequate oxygen supply or cerebral hypoxia, leading to brain tissue death, cerebral infarction, or ischaemic stroke. Ischemic stroke is a cause of long-term disability, and due to its high rate of oxygen consumption, the entire central nervous system is very sensitive to changes in oxygen concentration. We can model hypoxic brain injury using neural stem cells and perform hypoxic damage on the 3D model. After hypoxia injury, reoxygenation restores neuronal cell proliferation, but does not restore neuronal maturation. This study shows that human neural organoids

generated from neural stem cells provide new opportunities for the development of drug screening platforms and personalized modeling of neurodegenerative diseases, including hypoxic brain injury[26].

3.2. Toxicity testing and drug screening

Organoid models allow for the mass generation of human tissues and can therefore be used to explore the effects of compounds on disease phenotype or organ function. Using imaging and standard automated liquid handling, Organoid can therefore be adapted to standardized and well-established automated chemical screening pipelines. Many studies have taken advantage of this high-throughput screening system to find new drugs to treat diseases.[2] Notably, the formation of cultivation should be large-scale and automated. To achieve this, several protocols have been proposed using microfluidic or macrofluidic designs, including microspin culture systems and microcavity arrays. In addition, through the combination of robotics, large-scale cultivation and high-throughput processing can also be realized. Currently, neural organoids can be used in toxicology testing or drug screening[13].

3.2.1. Toxicity testing

iPSC-derived neural organoids can be applied to construct 3D host-pathogen interaction models that mimic virus-host interactions in the context of Zika virus (ZIKV) and herpesvirus contexts[14] .Zika virus is associated with severe neurological disorders (including Guillain-Barré syndrome) and congenital malformations (such as microcephaly). Recently, human neural organoids have been shown to mimic Zika virus replication and virus-mediated destruction of microcephaly neurogenesis, so they have been used to test the toxicity of potential drugs[14]. Herpesviridae is a heterogeneous group of viruses that are tropic to key organs such as the blood and vascular system, gastrointestinal tract, and nervous system, and can cause serious complications in seemingly healthy individuals. Herpes simplex virus (HSV) and cytomegalovirus (CMV) are two important herpes viruses that cause congenital infection and intrauterine growth restriction. Brain organoids derived from iPSCs have been used to mimic CMV and HSV infections in vitro. iPSC organoid cultures derived from CMV infection include viral genomes and show cytopenia, cysts, vacuoles, and necrotic regions. These results are similar to those observed in infected human clinical samples, reporting delayed neural maturation and cortical lamination abnormalities, demonstrating the valuable role of 3D culture in mimicking the CMV-induced pathology in developing brains in some ways[14].

3.2.2. Drug screening

The above material demonstrates that organoids are very effective in detecting neurotropics in viral infections, including Zika virus and SARS-CoV-2 virus[12]. In addition, stem cell-derived nerve cells can also be used as a platform for drug screening to identify therapies that block Zika virus infection or replication. Studies have shown that specific cell types are viral tropics, meaning that certain nerve cell types may be susceptible to infection. However, these in vitro studies must be validated by clinical evidence from patient-derived tissue samples. Several studies have now identified human organoids as an issue to better understand the vulnerability of specific nerve cell types to viral infections and to screen for potential therapeutic agents[12].

Another study showed that human neural organoids co-cultured with gas-solid chromatography (GSC) lineages from iPSCs and ESCs of different patient sources could show varying degrees of tumor aggressiveness. The organoid group exhibited high resistance to chemotherapy and radiation-induced genotoxicity compared to 2D cultures, suggesting the role of the 3D microenvironment in drug resistance[27]. To demonstrate the value of this system in chemotherapy drug screening, tumor organoids have been treated with EGFR inhibitors (tyrosine kinase irreversible inhibitors) that reduce tumor growth only in glioblastoma (GBM) organoids that overexpress EGFR[28].

4. Conclusion and Discussion

While neural organoid models provide valuable insights into specific aspects of human development and disease and promise to revolutionize drug development and clinical treatment protocols, there are still some hurdles to overcome: (1) heterogeneity and reproducibility. Reproducibility measures precision under reproducibility conditions, and since organoids are often heterogeneous in size, shape, and cellular composition, this heterogeneity can occur regardless of whether organoids come from different patients, are established in different laboratories, or even obtained from the same stem cells[23]. This lack of standardization creates enormous difficulties and reduces the use of organoids in disease modeling and drug discovery. Therefore, these procedures need to be further standardized and automated with media and starting cell types to reduce variability and improve system reproducibility. (2) Scalability. When used for drug screening, assays are typically performed in 384- or 1536-well plates, allowing sufficient control of study conditions to explore vast chemical spaces. However, organoids are typically generated in 96-well plates, which are too large for 1536-well plates and contain too many cells to be properly fed using 384 μL of medium in a 100-well plate. The implementation of rotating bioreactors to supply more organoids has partially overcome this hurdle, but limitations in drug screening remain. Today, engineering solutions using microfluidic systems, automated liquid handling, and robotic operations can try to overcome this problem and improve the scalability of drug screening methods[23]. (3) Maturity. The challenges of organoid research are also limited maturity, which can only form fetal-like tissue rather than adult tissue, and lack important physiological processes in the body as well as blood vessels, lymphatic vessels and nerve functions, and still cannot fully replicate human organs, so it can only be called a simple organ model[29]. Maturity may limit the potential of neural organoids to mimic adult-onset disease in vitro[23]. In addition, with the increase of the volume and complexity of organoids, due to the limitation of oxygen and nutrient surface diffusion, it often leads to hypoxia and death of internal cells, preventing the late development and maturation of organoids, thereby limiting their clinical application[29].

Current neural organoid technology is still imperfect, and there is still a need for improvement in terms of quality and quantity, as well as more accurate cell composition of stem cell-derived tissues. However, in general, most scholars at home and abroad are focusing on collaborating with other cutting-edge technologies (organ chips, micro-mobile arrays, high-throughput screening, etc.) to analyze and modify organoids, finely optimize and standardize each experimental step in the organoid culture system (medium components, cell types, quantity, reagent addition order, etc.), and overcome problems such as extracellular microenvironment, blood vessels, neural networks, maturity, etc., aiming to improve the stability, fidelity, reproducibility and scalability of organoids. We have reason to believe that with the continuous deepening and innovation of research, organoid models will gradually improve, and even give birth to new models and research tools, which will play an increasingly important role in translational medicine and clinical personalized treatment [2]. The continued development of organoid technology will further bridge the gap between basic research and translational medicine, bringing infinite possibilities for human development and disease research.

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