Review Article

Kafkas Üniversitesi Fen Bilimleri Enstitüsü Dergisi / Institute of Natural and Applied Science Journal

Cilt 18, Sayı 1, 31-36, 2025 / Volume 18 Issue 1, 31-36, 2025

Kafkas Üniversitesi Fen Bilimleri Enstitüsü Dergisi
Institute of Natural and Applied Science Journal

Dergi ana sayfası/ Journal home page: https://dergipark.org.tr/tr/pub/kujs

E-ISSN: 2587-2389

Development of Human Brain Organoids, The Formation of Neural Circuits and Relationship with Neurodevelopmental Diseases

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(İlk Gönderim / Received: 19. 08. 2024, Kabul / Accepted: 15. 07. 2025, Online Yayın / Published Online: 31.07.2025)

Keywords:
Stem cell,
Brain,
Tissue Engineering,
Regenerative Medicine,
Brain Organoids

Abstract: Human induced pluripotent stem cells (hiPSCs) and human embryonic stem cells (hESCs) are known as two types of human pluripotent stem cells (hPSCs) capable of self-renewing indefinitely in culture and differentiating into almost any type of human cell. Brain organoids, derived from pluripotent stem cells, serve as an extremely valuable resource for the scientific community. They are utilized to study the pathophysiology, tissue engineering, and development processes of human brains, distinguished by their ability to mimic natural growth mechanisms. In particular, human cortical organoids (hCOs) derived from human embryonic stem cells provide an excellent model for detailed three-dimensional investigations of human brain development and disorders. Despite advancements in this field, several unresolved issues persist. These limitations include non-functional blood arteries, restricted development of microglia, and the incomplete formation of the six distinct layers of the cortex. Additionally, the lack of microvascularization in modern hCOs limits the amount of oxygen and nutrients that can reach the inner regions of the hCOs. This review addresses the methods used for creating brain organoids and examines the challenges encountered in these processes. Furthermore, it highlights the latest advancements in the development of brain organoids and provides updated insights into their potential applications in studying early brain development and neurological disorders.

İnsan Beyin Organoidlerinin Gelişimi, Sinir Devrelerinin Oluşumu ve Nörogelişimsel Hastalıklarla İlişkisi

Anahtar Kelimeler:
Kök hücre,
Beyin,
Doku Mühendisliği,
Rejeneratif Tıp,
Beyin Organoidleri

Özet: İnsan kaynaklı pluripotent kök hücreleri (hiPSC'ler) ve insan embriyonik kök hücreleri (hESC'ler), kültür ortamında kendilerini sonsuza kadar yenileyebilen ve hemen her tür insan hücresine dönüşme potansiyeline sahip iki tür insan pluripotent kök hücre olarak bilinmektedir. Beyin organoidleri, pluripotent kök hücrelerden gelen, bilim dünyası için son derece değerli bir kaynak oluşturmaktadır. İnsan beyinlerinin patofizyolojisini, doku mühendisliğini ve gelişim süreçlerini araştırmak için kullanılmakta olup, doğal büyüme mekanizmaları taklit edebilme özellikleriyle dikkat çekerler. Özellikle, insan embriyonik kök hücrelerinden elde edilen insan kortikal organoidleri (hCO'lar), insan beyninin gelişimi ve bozukluklarını 3B dokuda ayrıntılı incelemek için mükemmel bir model sunmaktadır. Ancak, bu alandaki ilerlemelere rağmen, hala bir dizi cevapsız soru vardır. Bunlar arasında işlevsiz kan atardamarları, mikroglianın yetersiz gelişimi ve korteksin altı farklı katmanının tam anlamıyla oluşturulamaması bu modellerin sınırlılıkları arasında yer almaktadır. Bununla birlikte, modern hCO'larda mikro damarlanmanın olmaması, hCO'ların iç bölgelerine ulaşabilen oksijen ve besin miktarını sınırlamaktadır. Bu inceleme kapsamında, beyin organoidlerinin oluşturulma yöntemleri, bu süreçte yaşanan zorluklar ele alınmaktadır. Ayrıca, beyin organoidlerinin geliştirilmesi ve ilgili son yenilikler ve bu teknolojinin erken beyin gelişimi ile nörolojik bozuklukların araştırılmasındaki potansiyel kullanımları üzerine güncel bilgiler sunulmaktadır.

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DOI: 10.58688/kujs.1534932

1. INTRODUCTION

The technique of developing functional tissues utilizing cells, scaffolds, and physiologically active substances is known as tissue engineering. It originated from the field of biomaterials development and has since evolved into a multidisciplinary domain integrating biology, materials science, and engineering. Tissue engineering aims to create functional structures that conserve, heal, or augment damaged tissues or complete organs. As a crucial subfield of research within the broader field of regenerative healthcare, it seeks not only to replace lost or damaged tissues but also to enhance their functionality. Self-healing is another area of study in which the human body heals itself by regenerating its organs and tissues through its mechanisms, sometimes with the assistance of other biological substances. The phrases "regenerative medicine" and "tissue engineering" have become nearly identical as the profession aims to treat complicated, frequently chronic diseases (Contessi Negrini et al., 2021).

Due to its ability to support the culturing of cells in intricate 3D biomimetic frameworks, 3D printing is quickly becoming a potent instrument for tissue engineering. An increasing number of investigators in the field of biomedical engineering are using three-dimensional printing as a revolutionary tool in biomedicine, particularly for regenerative medicine and tissue engineering, due to the recent advancements in the technology. To reduce the need for animal testing and improve the reliability of test results, tissue engineering is a young domain that seeks to create replacements for natural human organs or tissues for laboratory medication testing or in vivo transplantation to lessen the scarcity of organs and the need for transplants (Thoma et al., 2014; Zhu et al., 2016). Possibly the most significant and intricate organ within an individual is the brain. Most scientists concur that the brain of an individual exhibits extraordinary potential concerning cognitive capacities and processes, such as language, thinking, recall, motor abilities, as well as other cognitive processes (Geschwind and Rakic, 2013). Numerous cell types, such as microglia, oligodendrocytes, astrocytes, and neurons, make up the human brain(Cakir et al., 2019). The brain of a person is one of the hardest organs to diagnose and treat (Azevedo et al., 2009). It is therefore very challenging to break down these interactions to determine which drive the pathogenic procedures and which are crucial for illness prevention (Jorfi et al., 2018). There is still much to learn to become knowledgeable regarding the intricacy of the human brain, both in terms of function and context. The absence of a model that accurately reflects the pathophysiological characteristics of the human brain is the primary obstacle to understanding it. Animal models, while valuable, often fail to replicate human-specific aspects of brain development and disease. Obtaining human brain tissue is challenging, and cultivated neural networks have a limited lifespan. Many of these restrictions are removed by the organoids made from human pluripotent stem cells (hPSCs), including embryonic stem cells and induced pluripotent stem cells (iPS) (Takahashi and Yamanaka, 2006).

Even today's scientists are fascinated by the cell, the smallest unit of a living entity that was originally seen by Robert Hooke in 1665. More than 200 different types of committed cells make up our body; some, like blood cells, function independently, while others form tissues and function in networks, such as the synapses that connect the brain to every part of the body. Despite their enormous diversity, all the cells in our body develop from a unicellular zygote. The zygote, the first embryonic stage of embryogenesis, undergoes mitotic cell division to become a morula, a blastocyst, and finally an implantation. The blastocyst's inner cell mass (ICM) develops into an epiblast in the post-implantation embryo before differentiating into the endoderm, mesoderm, or ectoderm, the three germ layers. In other words, the ICM can differentiate into any type of cell found in the human body. We call this extremely specialized capacity pluripotency. In the culture dish, pluripotency was initially introduced as embryonic stem cells (ESCs). Since ESCs may produce genetically modified mice, they have greatly benefited developmental biology (Liu et al, 2019). The sexual reproduction of an ovum results in the formation of a zygote, which is the first step in the development of a fertilized egg. The zygote's continuous multiplication or splitting multiplies the number of cells that are shielded from view by the zona pellucida, a glycoprotein barrier. When the zygote exits its fallopian tube and enters the morula phase (4–16 cells), it enters the uterus. The uterus gets stuffed with liquid in the center, known as the blastocoel, right after the morula enters. The blastocyst is the name for this stage in the developing embryo, which contains between 40 and 150 cells. The cells in the interior region of the blastocyst create the inner cell mass (ICM) or embryoblast, and the cells in the outermost region form the trophoblastic layer. A bilaminar embryo, made up of epiblasts adjacent to trophoblasts and hypoblasts next to the blastocyst cavity, is formed by the inner cell mass. The embryo's three germ layers—the endoderm, mesoderm, and ectoderm—are all derived from the epiblast (Moore et al., 2000). The pluripotent characteristic of ES cells originates from this core mass of cells. There are several types of stem cells. The collection of cells known as early ES cells is formed when the zygote divides before the blastocyst forms. Given their totipotent nature, these cells can differentiate into any type of cell found in the body. The cells in the ICM of a blastocyst are referred to as blastocyst ES cells. These cells possess the ability to differentiate into almost any type of cell found in the body because they are pluripotent fetal stem cells. The fetus's stem cells are in charge of every tissue’s initial development before delivery. Additionally, these cells have pluripotency. Umbilical cord-derived stem cells have the same genetic makeup as the infant. Due to their restricted ability to differentiate into distinct cell types, these cells are multipotent. These stem cells guide the growth and maintenance of already established tissues throughout an individual's life. These cells are also multipotent. Adult stem cells are commonly found in peripheral blood, nerves, skin, bone, and muscle. The unique characteristic of adult stem cells is their "plasticity," or the ability to differentiate into various types of tissue based on the growth or differentiation environment (Biswas and Hutchins, 2007). Stem cells are unique populations of cells that can differentiate into distinct lineages and undergo self-renewal processes. In 1981, the methods for cultivating mouse embryonic stem (mES) cells were first documented (Evans and Kaufman, 1981; Martin, 1981). The initial strains based on human embryonic stem

cells were created in 1998 (Thomson et al., 1998), while the first hES cell lines were created in 1994 (Bongso et al., 1994). Since then, technological developments have enhanced our understanding of ES cell proliferation and differentiation through the multiplication and manipulation of these cells (Biswas and Hutchins, 2007). The approach known as iPSC holds promise for addressing these problems. The therapeutic use of iPSCs was demonstrated in a mouse model of sickle-cell anemia in 2007. This genetic blood condition is caused by a β-globin gene deficiency. Gene repair using homologous recombination in mutant iPSCs made it possible to cure the illness in donor animals. This served as an excellent illustration of an iPSC-mediated regenerative medicine paradigm (Ohnuki and Takahashi, 2015). The easiest method for creating brain organoids with functioning neural networks is to fuse several organoids, such as those of the cortex, thalamus, and other regions, with different identities. Human pluripotent stem cells are used to create brain organoids, which self-assemble to create an organized structure made up of different progenitor, neural, and glial cell types, imitating the layout of the human embryonic brain (Jo et al., 2016; Kadoshima et al., 2013; Lancaster et al., 2017). When attempting to fuse organoids to replicate in vivo brain circuits, there are several factors to consider. The initial step is obtaining locally specified organoids as possible is the initial step. There are two main categories of brain organoid induction techniques. One technique promotes specific separation by adjusting cultural conditions, along with exogenous variables like growth factors or small molecules, while the other takes advantage of the inherent self-patterning characteristics of PSCs (Jo et al., 2016; Kadoshima et al., 2013). Controlling the directionality of connections between organoids is another important consideration. It may be necessary to create a single-directional axon growth between different organoids to replicate in vivo brain circuits. By utilizing the inherent variability of individual organoids, it may be possible not only to connect them but also to produce functioning neural networks within them. The natural self-organizing characteristics of aggregates formed from human pluripotent stem cells are employed in the culturing method developed by Knoblich's group (Lancaster and Knoblich, 2014). Using this method, aggregates are cultured without external stimuli for inductive patterning after being encased in Matrigel. The aggregates' internal signaling and behavior related to autonomous patterns are primarily responsible for their development into brain organoids. The development of polarity or axial data within organoids, and whether it can be controlled, are key questions in the production of arbitrarily designed organoids. The cellular variability of organoids stems, at least in part, from PSCs in a maintenance environment. Although it is generally known that experimental batches of brain organoids created using Knoblich's approach differ in some brain regions, it is unclear what modifications to PSCs during maintenance result in batch effects (Renner et al., 2017). The condition of proliferating cells and undifferentiated PSCs in growing organoids at the individual cell level will be analyzed to determine the causal relationship between heterogeneity in differentiating organoids and cellular states under maintenance settings. Managing the inherent capacity of organoids for self-patterning could benefit from this knowledge (Seto and Eiraku, 2019). Brain organoids derived from pluripotent stem cells are a valuable tool for studying the pathophysiology and growth of human brains because they are capable of imitating normal developmental processes. To a certain extent, the organoids derived from pluripotent adult stem cells, including induced pluripotent stem cells and embryonic stem cells, mimic cell growth and three-dimensional tissue cytoarchitecture in vivo (Camp et al., 2015; Kadoshima et al., 2013).

Consequently, hPSC-derived organoids represent a viable framework for studying both healthy and diseased human developmental processes (Jorfi et al., 2018). It is still difficult to use these organoids to examine the etiology of diseases associated with anomalies in connectivity between distant areas of the cerebral cortex, such as autism spectrum disorders (Dai et al., 2019). Constructing organoids with multiple brain areas and precise connections similar to those found in vivo is crucial in this regard. hiPSCs are readily available sources of stem cells from both healthy individuals and those with brain disorders. As a result, neural cells produced from hiPSCs have been utilized in drug discovery and studies on diseases of the brain, such as studies on Alzheimer's disease, Parkinson’s disease (PD), and autism spectrum. Since many brain disorders have different etiologies and are complicated by varying genetic and epigenetic backgrounds, it is possible to explore the unique pathophysiological characteristics of each disease using patient-derived hiPSCs. An exceptional opportunity to simulate human brain growth and activity is provided by brain organoids, which are commonly unattainable through in-person testing, because they mimic the cellular framework of the human brain as well as its overall tissue organization and growth pattern, unlike traditional 2D cultures of cells (Qian et al., 2019). The hCOs have made it possible to investigate neurological conditions and the early stages of brain growth exclusively (Pașca, 2018; Pașca et al., 2015; Qian et al., 2016). While the development of 3D brain organoids has opened up new possibilities, there are still a number of unanswered questions. These include the absence of functioning blood vessels, the limited development of microglia, and the complexity of different cortical layers (Heide et al., 2018). It is generally accepted that the absence of functioning vasculature impairs the development of neuronal progenitor cells (Shen et al., 2004). The use of brain organoids has advanced the modeling of monogenic diseases in humans (Birey et al., 2017). In addition to complex idiopathic disorders like Autism Spectrum Disorder (ASDs) (Mariani et al., 2015). A notable increase in neural progenitor cell division was observed in brain organoids generated from iPSCs of patients, using an earlier selected endophenotype of ASD patients with macrocephaly (Trujillo and Muotri, 2018). Due to the complexity of the human brain and the restricted access to human brain tissue, it is difficult to understand the neurological causes and mechanisms of many brain illnesses. Our understanding of the effects of genetic alterations and external factors on neural development, neural circuit formation, and function has significantly increased due to studies conducted on model organisms, particularly rats (Leung and Jia, 2016). However, many aspects of human brain development and disorders differ from those observed in rodent systems due to the significant evolutionary distance between the mouse and human brains, as well as the larger size and complexity of the primate brain (Zhao and Bhattacharyya, 2018). hESCs and hiPSCs have the potential

to generate a variety of human cell kinds *in vitro*, such as neurons and glia, enabling the direct study of biological processes in a human context. These protocols for the culture and differentiation of hPSCs have made this possible (Avior et al., 2016). Studying the effects of genotypes associated with health and disease on the physiology and development of human cells has been made possible in part by hiPSC technologies. Moreover, summarizing alterations associated with diseases cultured cells and correcting genetic defects in hiPSCs generated from patients have been made easier by genetic modifications using CRISPR-Cas9, TALENs, and zinc finger nucleases. In 2015, Hotta and Yamanaka's methods greatly advanced the study of humans from these methods, neurobiology, revealing cellular characteristics associated with brain diseases.. Sterneckert and colleagues (2014) have shown, however, that monolayered culture methods can only reproduce and study a limited number of disease phenotypes due to the absence of tissue architecture and tissue environment. A new area of study was made possible by the original brain organoid culture methods developed by the Sasai group (Kadoshima et al., 2013; Lancaster et al., 2013; Seto and Eiraku, 2019). Many brain organoid protocols and associated methods, including spheroid protocols (Paşca et al., 2015), have been developed in recent years to replicate certain features of prenatal and embryonic neural growth. The breadth of existing protocols includes the creation of simpler organoids that are targeted at particular brain regions, as well as more complex organoids that replicate connections between cells and interconnectedness among various brain regions.

Brain organoids: a collection of bioassays to study neurodevelopment and illness in humans, and neuroectodermal fate to produce neural tissue. Lancaster et al. developed the brain organoid procedure using sparse media based on this concept. With the help of matrix embedding and hPSC self-organization and self-patterning, this protocol produces neuronal structures made of neuroectodermal tissue. Recapitulating the structure of the brainstem and progenitor cells' germinal zones structure of tissues as the distinct neurons migrate outward. Thus, this procedure can be used to study phenotypes associated with tissue topology. For example, the cerebral organoid procedure was recently used to mimic periventricular heterotopia using hiPSCs from an individual with variations in the FAT4/DCSH1 genes. In examining the effects of the FAT4/DCSH1 variations, the authors discovered a deviation in the radial glia cells, which serves as a support for the cortical neurons' radial migration. Furthermore, the mutant organoids exhibited altered neuronal condition of neurons with differently generated genes linked to movement and guidance of neurons, as demonstrated by RNA sequencing analysis combined with pedigree restoration. The discovery that some neurons in the mutant organoids had abnormal migration patterns supported this finding (Klaus et al., 2019). Differential signaling pathways that are either triggered or suppressed by signaling molecules or morphogens released *in vivo*, which guide the arrangement of cells in specific areas to design the neural tube during development, establishing the identity of distinct brain regions. By including molecules with specific patterns in the culture media that either promote or inhibit signaling for growth at the appropriate stages, one can use these guidelines *in vitro* to control organoids and guide the cells to adopt the fates of specific brain regions. Although these chemical combinations define the general identities of brain regions, it is difficult to create organoids that are specific to individual subregions. For example, the visual cortex, located in the occipital lobe, and the prefrontal cortex, which is located in the frontal region, for example, are two examples of the various functional areas that make up the mammalian cortex. These areas vary in terms of their cellular diversity, gene expression patterns, and functions (Clowry et al., 2018; Molnár et al., 2019). Furthermore, human brain regions, such as the prefrontal cortex, are abnormally large and linked to mental illnesses. Nevertheless, the current cortical organoid methods produce cortical tissues with heterogeneous identities. Innovative techniques that produce organoids of particular cortical regions will be valuable in advancing research on human brain disorders.

Accordingly, it has been demonstrated that applying FGF8, an anterior developmental factor, reaches the cortex organoids causes the cortical tissue to rostralize (Kadoshima et al., 2013). However, since identical signaling molecules are involved in the arrangement of distinct areas of the brain, the present task is to find molecular mixtures characterized by chemical components or corresponding treatment timelines that reliably and economically identify specific brain regions of interest. The brain's intricate circuitry is the result of interactions between cells in various, frequently very distant, brain areas. Two different cellular actions help overcome the issue of selecting the ideal functional partner: As an example, neurons in the cortex migrate forward from the ventral telencephalon into the brain's cortex. First, aggressive cell migration to the desired locations and second, long-term projections; for example, the "thalamocortical tract" relays sensory data from neurons in the cortical and thalamic nuclei (Molnár et al., 2019). Axon pathfinding or neuronal migration defects can cause severe birth defects and cortical abnormalities. Engel (2010) and Ross & Walsh (2001). Nevertheless, region-specific organoids are unable to recreate interregional cell interactions, and the inconsistent cerebral organoid methodology hinders the establishment of consistent areas of expertise connected. To get around this issue, several labs created organoid fusion techniques, in which fresh organoids and prepatterned EB are mixed in a tube or encapsulated in a single Matrigel droplet (Birey et al., 2017). The two regions eventually merge and show intricate cell connections. Studying the impact of genetic background on neuronal behavior, both cell autonomous and nonautonomous, is made possible by the fusion of organoids with disparate genetic origins. Furthermore, viewing of these intricate cellular behaviors, like long-term estimates, axon navigation, and migration of neurons, is made possible by Direct observation of the organotypic segments or merged organoids. Morphogen activity gradients in the AP and DV directions are responsible for the brain's immense neuronal diversity. In 2015, Suzuki and Vanderhaeghen. Any morphogen that is supplied to the media, though, is given in a consistent concentration, which might limit the range of outcomes. Employing a two-stage EB generation procedure, Cederquist et al. devised a unique method for creating organoids with downstream patterning and a gradient of morphogen activity. Originally, transgenic hPSCs that could induce the production of a morphogen were used to create a small EB. A larger EB with an inducible signaling center or

organizer was formed by the subsequent seeding of more hPSCs. The released morphogen spread throughout the tissue to form a gradient once morphogen expression was induced. Cederquist et al. produced organoids having telencephalic function and a radially organized DV axis by using this approach to construct a range of the morphogen SHH that induces ventral growth. Cederquist and colleagues, 2019. Microglia and vascular cells are examples of non-neurodermoectodermal origin cells seen in the brain. Macrophages found in the brain, called microglia, are essential for the development and operation of neurons. Neurodegenerative diseases and mental conditions, including autism and schizophrenia, are linked to microglial dysfunction. However, in the current organoid systems, it is challenging to create microglia synchronously with the neuroectoderm, since they originate in the yolk sac. According to a new study, cerebral organoids contain microglial-like cells that arise from a small number of mesodermal cells during the cerebral organoid process. Nevertheless, other researchers have co-cultured organoids with hiPSC-derived microglia, allowing the microglia to penetrate the organoid in order to broaden the application of these brain organoid procedures. Recently, the removal of amyloid β protein clumps containing mutations linked to familial Alzheimer's disease was investigated using this co-culture paradigm. *In vitro* restitution of microglial activities in organoids will be enhanced by further advancements and experiments with these techniques. The migration of cortical interneurons is a particularly illuminating subject for the organoid fusion technique. It is now feasible to observe the distinctive movement of cortical neurons in salt-dependent motion and investigate the effects of both genetic and pharmacological perturbations on these migrant characteristics, thanks to the convergence of ventral and dorsal organoids, or DV mixtures. The effects of mutations in functions causing Timothy syndrome (TS) were investigated by Birey et al. using the DV mixture method. Upon fusing control dorsal telencephalic organoids with ventral organoids cultivated using hiPSCs generated from TS patients, the interneurons harboring the TS mutation displayed abnormalities in saltatory movement that were cell-autonomous. The effects of the alterations were mitigated, and the issues were resolved by using a substance that decreased the calcium channel's function (Birey et al., 2017). Though still in its infancy, the field of brain organoids is growing thanks to the development of improved techniques. Brain organoid techonology possesses the potential to create practical assays for examining additional complicated characteristics when combined with novel experimental techniques.

2. CONCLUSION

This review has highlighted several recent studies that point to potential directions for future research using brain organoids to study brain diseases and functions within the context of neural networks. Brain organoids have emerged as a highly valuable tool for understanding the developmental processes of the human brain and the underlying mechanisms of neurological disorders. Derived from hiPSCs, organoids mimic natural growth mechanisms, providing researchers with the opportunity to study early brain development and pathophysiological processes in a three-dimensional environment. Specific models, such as hCOs, hold significant potential, particularly in understanding the formation and functions of cortical layers.

However, current technologies face several limitations. Recent innovative studies have introduced various strategies to overcome these limitations. Techniques such as microvascularization technologies, advanced bioengineering approaches, and the incorporation of extracellular matrices into the organoid environment aim to enhance the accuracy of these models.

In the future, this technology is expected to pave the way for groundbreaking applications in neurological disorder treatment, early diagnosis, and personalized medicine. Further optimization of brain organoids holds immense potential for both basic science and clinical research. In this context, interdisciplinary collaborations and continuous technological advancements will solidify the role of brain organoids in scientific investigations.

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