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Advancing Towards Physiologically Relevant Models of the Brain: Three-Dimensional Human Induced Pluripotent Stem Cell (hiPSC)-Based Cell Culture Systems in Neuroscience

Martina von der Bey[1,2], Ebru Ercan-Herbst[1]*

[1]BioMed X Institute, Im Neuenheimer Feld, Heidelberg, Germany

[2]Molecular and Translational Neuroscience, Department of Neurology, Ulm University, Albert-Einstein-Allee, Ulm, Germany

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***Correspondence:**

*Dr. Ebru Ercan-Herbst, BioMed X Institute, Im Neuenheimer Feld, Heidelberg, Germany; Email: ercan@bio.mx

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ABSTRACT

One of the biggest challenges in the field of neurological disorders is the limited availability of freshly dissected human brain tissue. Therefore, the use of human induced pluripotent stem cells (hiPSCs) is important to develop human brain-like models to study the interaction of different brain cell types in health and disease. For physiologically relevant disease modeling, three-dimensional (3D) cell culture systems are of great importance because they provide a more representative *in vivo*-like micro-environment to the cells. The field of 3D cell culture systems using diverse hiPSC-derived cells is growing and gets steadily advanced. However, to this day, there is no cell culture model available that includes all brain cell types. Here, we review the latest improvements of 3D hiPSC-based cell culture systems in the field of neuroscience. We focus on innovations for the generation of neurons, astrocytes, oligodendrocytes, microglia as well as endothelial cells and pericytes.

Introduction

Cell culture systems are an important *in vitro* tool in basic research and essential in drug discovery and development. The first cell culture experiment in history was performed in 1907 by Harrison and colleagues[1] who developed a method to directly observe the growth of a nerve fiber cell. This innovation founded a new research field allowing the observation and study of growing and differentiating cells outside of an organism. Since that time, cell culture methods have been greatly advanced, and the two-dimensional (2D) cell culture systems evolved to be the standard *in vitro* tool. These 2D cell culture systems have already provided many insights into basic cellular functions, biological mechanisms and various disease processes. Especially in drug discovery and development, 2D cell culture systems are essential concerning compound testing and high-throughput screening (HTS) assays. However, 2D cell cultures do not represent the physiological *in vivo* microenvironment of the cells[2]. Usually, a homogenous cell population is cultured as a monolayer, while *in vivo* the cells are interacting with a heterogenous cell population. In addition, in physiological conditions the cells are interacting with multiple extracellular matrix (ECM) components, which can actively affect the behavior of the cells[3]. In fundamental and preclinical research, one of the biggest challenges is still the establishment of physiologically relevant *in vitro* models. To address this challenge, multiple three-dimensional (3D) cell culture systems have been developed. These 3D cell culture systems range from scaffold and scaffold-free techniques to more complex systems like organoids. Scaffold-based systems can create a 3D microenvironment, for example, by a network of nano- and microfibers[4,5] or through

hydrogels[6,7]. Spheroids and complex organoid models allow the formation of self-assembled cell aggregates recapitulating the cellular organization and functionality of specific tissues or organs[8-10]. These different 3D cell culture systems provide a more representative *in vivo*-like microenvironment which influences cellular features like morphology, proliferation, differentiation and migration[11,12]. In drug discovery and development, 3D cell culture systems are more frequently used in HTS assays[13,14]. Recently, the U.S. Food and Drug Administration (FDA) modernization act 2.0 (2022) marked an advancement encompassing that the FDA is now allowed to approve biological products and drugs that have not been tested in animal models[15]. This milestone opens the door to a potential increase in cell-based models within pharmacological research in the coming years.

3D human induced pluripotent stem cell (hiPSC)-based cell culture systems in neuroscience

Limited availability of human brain material poses a significant challenge in the field of studying neurological disorders. Post-mortem brain tissue provides a valuable source to study neuroanatomy and to perform molecular and neuropathological analysis[16-18]. Nevertheless, this material lacks information about functionality of cells which can only be examined with living cells. Therefore, the hiPSC technology is an essential tool to develop human brain-like models. This technology enables the differentiation of the brain cell types of interest, also from diverse patient-derived material. For physiologically relevant disease modeling it is important to consider all cell types present in the human brain. This includes different subtypes of neurons, astrocytes, oligodendrocytes, microglia as well as endothelial cells and pericytes. To date, there is still no existing cell culture model that encompasses all the above-mentioned cell types. This is primarily due to the difficulty in identifying the optimal culturing conditions necessary for the survival of each individual cell type. This mini-review will provide an overview of protocols for the generation and differentiation of these brain cell types in 3D cell culture systems that have been steadily advanced and optimized over the last years.

Neurons and Astrocytes

Neurons are electrically excitable cells and the primary unit of the central nervous system (CNS). Astrocytes execute numerous functions to maintain homeostasis and ensure proper neuronal signaling. They support neurons, for example, by providing nutrients, regulating ion concentration and they participate in blood-brain barrier formation[19]. These two cell types interact very closely with each other. For small molecule-induced hiPSC-based neuronal differentiation protocols, astrocytes are often generated in addition to ensure proper neuronal maturation[4]. Sato and colleagues[20] developed a feeder-free culture system for the generation of region-specific and high-purity neuronal cultures. The overexpression of the transcription factor neurogenin 2 (*NGN2*) can result in a heterogeneous neuronal cell population[21,22] without the need of supporting astroglial cells. These systems can generate mature neurons faster[21] compared to the small molecule-induced systems, but may lead to neuronal subpopulations more resembling neurons from the peripheral nervous system[22]. Co-culture models of neurons and astrocytes were established and advanced during the last years. The culturing of hiPSC-derived neurons and astrocytes in 3D systems includes scaffold-based[23] and scaffold-free systems[24]. As an example, Park and colleagues[24] generated a 3D spheroid model with hiPSC-derived neurons and astrocytes which is reproducible in regard to spheroid size and cell type composition which is of advantage for high-throughput drug efficacy screenings. Functional assays, like electrophysiological recordings, have a big impact in translational research in order to understand neuronal dynamics in health and disease. From traditional patch-clamp techniques[25] to high-density microelectrode arrays[26], single cell as well as network recordings can be performed nowadays revealing insights into cell communication. These techniques are already highly advanced for 2D systems and are recently improving for 3D applications[27].

Oligodendrocyte lineage cells

hiPSC-derived oligodendrocyte lineage cells are often not included in co-culture systems, although they play an important role by myelinating and supporting neurons[28]. One reason might be that optimized protocols are limited and, in comparison to neurons, oligodendrocyte lineage cells develop over several intermediate stages[29]. In this regard, the generation and differentiation of highly mature and functional oligodendrocytes is challenging and takes a long time, especially if the differentiation is performed by mimicking the natural environment with small molecules[30]. One of the most frequently used protocols in this field was developed by Douvaras and Fossati[31] resulting in mature oligodendrocytes after 75 days *in vitro*. We recently published a manuscript showing that this original protocol can be translated to a 3D cell culture system generating functional and myelinating oligodendrocytes[4]. Our protocol describes a scaffold-based 3D co-culture system of hiPSC-derived oligodendrocyte lineage cells and cortical neurons on nanofibers[4]. Ehrlich and colleagues[32] also developed a protocol for the rapid and efficient generation of oligodendrocytes on aligned nanofibers. For a faster generation of myelinating oligodendrocytes they used the overexpression of specific oligodendrocyte lineage cell-directing factors, like SOX10 and OLIG2[32], which can be nowadays also applied for organoid systems[33]. There

are several protocols available to study oligodendrocytes with neural spheroids and organoids containing neurons, astrocytes and oligodendrocyte lineage cells[33-37]. For example, Kim and colleagues[37] could show that organoid models reveal human oligodendrogenesis with ventral and dorsal origins by generating fused forebrain organoids. The development of a compact and multi-layered myelin sheath is of importance for functional readouts of myelination efficiency. To date, there is only one model with spinal cord-patterned myelinating organoids showing proper myelination after several months *in vitro*[34]. These different co-culture systems display useful disease modeling tools to study myelination biology in more physiologically relevant conditions. Nevertheless, for studies depending on mature and myelinating oligodendrocytes, time is the limiting factor which might impair the usability of the existing models for HTS assays.

Microglia

Microglia are the immune cells of the CNS fulfilling multiple tasks to support the developing brain as well as to maintain homeostasis[38]. The majority of the brain cells originates from the neuroectoderm, while microglial cells arise from the mesodermal germ layer with hematopoietic origin[39]. During early development, microglial precursor cells are migrating and infiltrating the CNS via the blood stream[40]. Therefore, cells of the microglial lineage need to be generated separately from neurons and the other glial cells (astrocytes and oligodendrocyte lineage cells) and afterwards introduced into the culture system. One approach is to integrate hiPSC-derived macrophage progenitors into organoids which are able to develop into mature microglial cells recapitulating cell-specific features[41]. Another approach was described by Xu and colleagues[42] who developed a system to generate cerebral organoids where the microglia cells were able to phagocytose and prune synapses. It is important that microglia are integrated more frequently into co-culture systems because of their function as immune cells[38]. However, the proper maturation of these cells seems to be dependent on a supporting brain environment[43]. That is why there are still challenges to overcome, like the short-term survival and the absence of the expression of environment-specific factors[43]. Nevertheless, these cells have a great influence on the behavior of other brain cell types which brings them more into focus for neurological diseases[44].

Endothelial cells and Pericytes

Endothelial cells are the innermost layer that coats the interior walls of blood vessels directly contacting blood components[45]. Pericytes are mesenchymal-derived cells located within the capillary basement membrane close to astrocytic endfeet[46]. In the brain, these cells fulfill a special role because they form the blood-brain-barrier (BBB). This is of vital importance regarding the maintenance of the brains' micro-environment ensuring proper cell function[47]. Since the developing CNS does not naturally generate their progenitor cells, their integration into the model system becomes vital for proper function and maturation of different brain cell types[48]. Campisi and colleagues[49] developed a 3D BBB model in fibrin gel exhibiting perfusable and selective microvasculature. Their microfluidic system comprises hiPSC-derived endothelial cells, pericytes and astrocytes as a self-assembled vascular network. There are also different approaches to introduce vascularization into organoid systems. Cerebral organoids can be generated with a mix of hiPSCs and human umbilical vein endothelial cells (HUVECs) which supports the maturation of neurons[50]. Sun and colleagues[51] could generate organoids that showed structures similar to the BBB. Therefore, they fused one brain organoid with two blood vessel organoids resulting in the generation of a vascularized brain organoid with selective permeability to molecules with different BBB penetration capabilities. They were able to reproduce some features of the BBB, but the tube-like structures of the blood vessels were not entirely formed[51]. Models including vascularization are essential tools for drug development in the field of neurological disorders because drugs need to pass the BBB to reach their target location.

Limitations of 3D hiPSC-based cell culture systems

3D cell culture systems are important for the generation of human-like cell culture models that can recapitulate physiological conditions by proper interaction of diverse brain cell types. Although these systems have advanced steadily over the last years, there are still some limitations. To this day, most automated analysis techniques/machines are built for 2D cell culture systems. There is a high need for advanced techniques applicable also for 3D cell culture systems, from applications like microscopy techniques to upscaling for automated generation and culturing of cells and HTS assays. In addition, more focus should be placed on functional readouts. Electrophysiological readout techniques, especially microelectrode arrays, are becoming more popular because user-friendly and easy-to-handle devices are being commercialized. Although these systems can now also be used for 3D structures, such as organoids[27], there is still the limitation that only the area where the organoid touches the electrodes is recorded. It is not possible to record the electrical signals from the whole organoid[52].

One other limitation especially for organoids, is the insufficient oxygen and nutrients supply and thus the development of a necrotic core, which is due to the lack of a properly functional vascularization system[53].

Conclusion

The field of 3D cell culture systems using diverse hiPSC-derived cells is growing and gets steadily advanced. However, there are still limitations and challenges to take to get closer to the best human brain-like model which is essential for disease modeling. As of today, achieving a complete representation of a neurological disease in a model is nearly impossible. Consequently, researchers are compelled to focus on individual aspects of a disease in order to establish disease-like models. Nonetheless, there remains a need for the development of cell culture models that incorporate all types of brain cells. Considerable efforts are currently being dedicated to advancing and refining these systems. The goal is to create functionally advanced, uniform and more standardized 3D cell culture systems that can be of great advantage for disease modeling.

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