Chapter

# Applications of Neural Organoids in Neurodevelopment and Regenerative Medicine

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## Abstract

Recent advances in stem cell technologies have enabled the application of three-dimensional neural organoids for exploring the mechanisms of neurodevelopment and regenerative medicine. Over the past decade, series of studies have been carried out to investigate the cellular and molecular events of human neurogenesis using animal models, while the species differences between animal models and human being prevent a full understanding of human neurogenesis. Human neural organoids provide a new model system for gaining a more complete understanding of human neural development and their applications in regenerative medicine. In this chapter, the recent advances of the neural organoids of the brain and retina as well as their applications in neurodevelopment and regenerative medicine are reviewed.

**Keywords:** neural organoids, neurogenesis, brain, retina, neurodevelopment, regenerative medicine

## 1. Introduction

Researchers have been attracted by the mystery of human neural development for hundreds of years. Numerous cellular and animal models have been explored to improve our understanding of neurogenesis in humans for hundreds of years. Although animal models have greatly improved our understanding of neural development, neurological disorders, cortical architecture, and functional regionalization, there are significant differences between the human and rodent brains. For example, the organization and behavior of neural progenitors during embryonic development determine the expansion and folding of the human neocortex to a large degree. Therefore, studying the development of the human brain requires models with human brain characteristics. Organoids are simply, self-organized three-dimensional (3D) tissue cultures that are derived from human pluripotent stem cells (hPSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), which has gained great interest in simulating tissue development and disease. This technology opens a window to observe some of the most elusive aspects of human biology. Compared with animal models or two-dimensional (2D) cell culture systems,

3D-cultured organoids can overcome the differences between species and closely represent the realistic human-specific development features, which can be utilized to mimic the architecture and functionality of the human tissues, having great advantages in explaining the unique human developmental processes [1, 2]. In the field of neurodevelopment and regenerative medicine, neural organoids replicate human specific features of neurodevelopment, contributing to modeling neurogenesis and neurological diseases [3, 4]. Central nervous system (CNS) injury or damage initiates spatial and temporal neurodegeneration, resulting in irreversible neuronal loss and functional deficits. The vertebrate retina is an extension of the CNS that is composed of seven main types of neurons and glial cells. In recent years, emerging organoid-based research studies of brain and retina have made progress in understanding neural organogenesis, which facilitates successful application of 3D organoid systems in disease modeling and regenerative medicine. In this chapter, we summarize the application of neural organoids of the brain and retina in neurodevelopment and regenerative medicine.

## 2. Organoids in neural development

CNS is generally regarded as the most complex system in human body. Limited by accessibility of living neural tissues and ethical challenges, human-specific features of neurodevelopment and neurological diseases remain largely unknown to us. Recent advances in stem cell technologies and 3D culture neural organoids have opened a new avenue in exploring the mechanisms of neurodevelopment. Early versions of the neural organoids range from complex neural epithelial structures to disorganized brain regions with large cellular diversity [5]. By supplementing exogenous factors and assembly of organoids during embryonic brain development, efforts have been made to gain the well-developed multilayer neural organoids and higher-order functions in terms of controlling patterning, morphogenesis, and function [6, 7].

### 2.1 Neural organoids in brain development

Through the embryonic brain development, neural progenitors progressively follow precise orchestration and coordination to acquire their spatial identities, a process characterized by successive changes in cellular composition and cyto-architecture (**Figure 1a**). Dysregulation of this process may affect neurogenesis, synaptogenesis, and myelination and induce neurological or psychiatric disorders. To better investigate the early formation and function of the human brain *in vitro*, two different methodologies have been applied to generate human brain organoids: unguided and guided methods. The unguided methods rely largely on the capacity of spontaneous morphogenesis and intrinsic differentiation of the 3D aggregates while the guided organoid methods highly require supplementation of exogenous factors to induce hPSCs to differentiate toward specific brain regions [5]. Over the past decade, guided methods were induced by a set of growth factors and small molecules to induce the production of brain organs containing broad and specific identity, including forebrain, large cortical organoids, cerebellum, midbrain, and hippocampus. For instance, glycogen synthase kinase-3 (GSK-3) inhibitor, SMAD inhibitors, and WNT3A were for forebrain organoids induction [6]; SMAD inhibitors, Wnt activator, sonic hedgehog (SHH), and FGF8 were used for midbrain organoids induction [8]; FGF19 and SDF1 for cerebellum organoids induction [9]; WNT-3A and SHH were

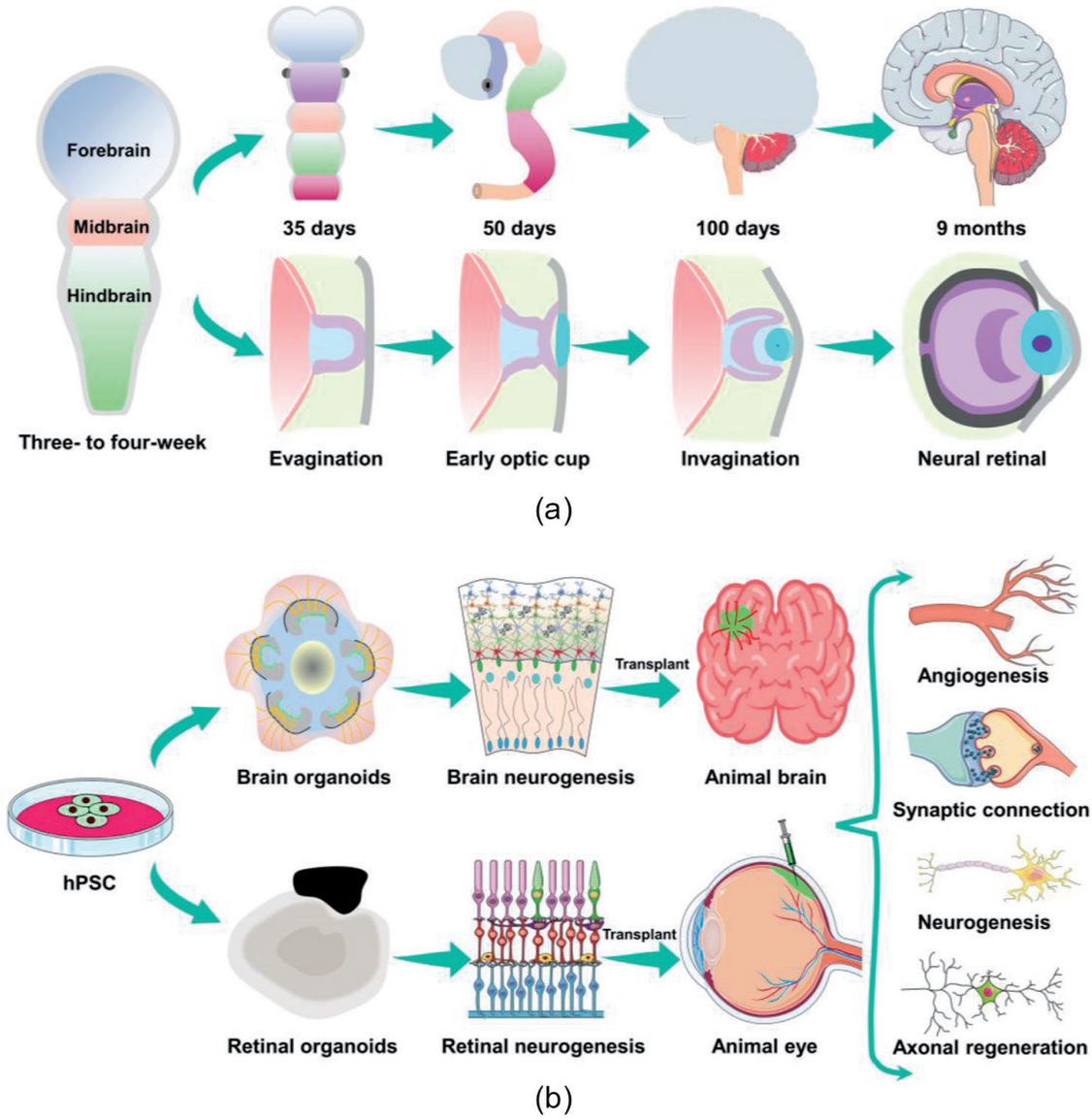


**Figure 1.**
*Schematic depiction of brain and eye development in vivo and in vitro. a. The timeline of brain development in human body and the features of human retinal development. b. Generation of hPSC-derived human brain organoids and retinal organoids in vitro, and the application of the generated organoids in regenerative medicine.*

used for hypothalamic organoids induction [10]. These generated brain organoids show robust neuronal and glial subtypes resembling the organization, transcriptional frameworks, and developmental timing of a primitive human fetal brain.

The 3D cultured brain organoids have been proven useful for many applications in basic research, for example, the development of the human brain cortex. It firstly begins with the expansion of the neuroepithelium, and then folds into six different layers. The main principles of the cortical layer formation are similar between mammals, such as primates and humans; however, the morphological differences are unneglectable. It is well known that neuronal number in primates massively increases in cortical surface, which eventually leads to the gyrification of the cortex (the generation of gyri and sulci) [11]. However, the mechanisms controlling the generation of gyrification are still not clear during the formation of cortical areas. The application of cortical organoids has helped us better understand the rapid expansion of human neocortex and the formation of cerebral cortical sulcus and gyrus. Karzbrun et al. revealed two opposing mechanical forces with the usage of

the brain organoids-on-a-chip: the middle cytoskeletal contraction and peripheral cell-cycle-dependent nuclear expansion, physically leading to differential growth and folding into brain wrinkling [12], and the extracellular matrix (ECM) components are implicated in neocortical expansion [13].

For another example, brain organoids are used to investigate the development of cellular interactions in the human brain. The human CNS originated from several distinct vesicles and then after a range of progenitors migrate and integrate, it moves into areas to generate multiple intertwined regions, ultimately resulting in emerging complex networks, neurons branching, and projecting. To model the intricate cellular interactions in human brain, fusing regionalized organoids into assembloids recapitulates more elaborate biological processes of brain development. This approach has been applied to study forebrain axis establishment, interneuron migration, oligodendrogenesis, and neuronal projections like the fused dorsal-ventral cerebral organoids to model interneuron migration in [7, 14, 15].

## 2.2 Neural organoids in retinal development

The eye originates from the ventral diencephalon, where a group of eye field transcription factors (EFTFs) are highly expressed such as PAX6, RAX, SIX3, and OTX2, and becomes specified as the eye field [16]. The eye field region is firstly split into optic vesicles in pairs and subsequently forms the optic cup by experiencing the valgus and invagination of the optic vesicle. The outer layer of the optic cup develops into retinal pigment epithelium(RPE) while the inner layer develops into neural retina. The neural retina with multilayered structure undergoes different phases of development, with different types of cells differentiating and maturing over the time (**Figure 1a**).

However, the understandings of the function and development of the human retinal are limited by scarce human fetal retina sample and species differences between animals and human. Since 2011, Sasai et al. released a landmark study to generate a self-organized 3D optic cup with layered neuroepithelia from mouse pluripotent stem cells (mPSCs), which opened a window for generating retinal models [17]. Many research groups have subsequently optimized the protocol to generate human retinal organoids derived from hPSCs. During retinal organoid generation, stem cell patterns the eye field-like regions expressing a complete component of the EFTFs to mimic the optic vesicle in early development [18]. What is encouraging, these tiny retinal organoids even contain almost all relevant retinal cell types: retinal ganglion cells (RGCs), amacrine cells, horizontal cells, bipolar cells, Müller cells, and photoreceptors.

### *2.2.1 Retinal organoids in RGC development*

RGCs, the early-born neurons, transmit visual information between the eye and the brain, playing a critical role in retinal neuronal outputs. The loss of RGCs trends to result in a group of degenerative diseases such as glaucoma and optic nerve hypoplasia. Due to the specific time point of the RGC development, it is a challenge to obtain human fetus samples. In addition, the long-distance projection of neurites is the mostly important characteristic for RGC development as the extension of axons is regulated by extrinsic factors, including the ECM, growth factors, and glial cells. Recent several approaches have improved the capacity to differentiate hPSC-derived retinal organoids into RGCs that possess appropriate morphological and functional properties [19]. For example, Fligor et al. found that substrate composition including

laminin and Matrigel shows the most conducive for RGC neurite outgrowth; similarly, the growth factors with Netrin-1 and BDNF have the ability to guide and direct RGC axons outgrowth [20]. Besides, single-cell RNA sequencing (RNA-seq) results proved that the ganglion cells of retinal organoids at day 60 give the similar clusters to the human fetal retina on day 59 [21]. Collectively, the established retinal organoids serve as effective models for investigations of RGC development and disease modeling and as a valuable tool for cell replacement.

*2.2.2 Retinal organoids in photoreceptors development*

Rod and cone photoreceptors are specialized neurons with functioning in the initial step of vision, which convert light stimuli into neurological responses. Rods are highly sensitive to light and operate under dim lighting conditions while the cones control color vision and high visual acuity. It is reported that the progressive loss of photoreceptors leads to blindness-associated inherited retinal diseases(IRDs), such as well-known retinitis pigmentosa (RP), congenital stationary night blindness(CSNB), and Leber congenital amaurosis (LCA). Therefore, it is particularly important to understand retinal progenitor fate choices toward rod photoreceptors and cone subtypes during retinogenesis. As such, the phototransduction mechanism requires a complicated cascade of gene regulatory networks. Aiming to induce hPCS-derived retinal organoids with mature photoreceptors, efforts of genetic manipulation and transcriptomic analysis have become the focal point for researchers [22]. Most recently, NRL (neural retina leucine zipper)$^{-/-}$ human-based 3D organoids were used to uncover the regulative role of MEF2C in cones' development [22]. RNAseq analysis of hPCS-derived retinal organoids has identified certain molecular signatures related with human photoreceptors development [23]. In brief, these observations and datasets have enabled to reconstruct developmental trajectories and recapitulate dynamics *in vivo* photoreceptors development.

## 3. Neural organoids in regenerative medicine

Neural organoids, which recapitulate the process of native neurogenesis in the development of CNS, have been applied in a large variety of areas including simulating brain development and retinogenesis. Moreover, emerging organoid-based cell transplantation has made considerable progress in reconstruction of lost neural circuits, damaged neural cortex and visual function, which facilitates the application of 3D organoid systems in disease modeling and regenerative medicine. Representative examples are involved in two aspects: (a) isolating neural progenitor cells (NPCs) from neural organoids; (b) transplanting neural organoids in immunodeficient animals. The stem cells in the organoids derived from hPSCs present a higher survival rate and closer connection with the surrounding tissues in the host. Distinct from conventional stem cell therapy usually focusing on specific populations of stem cells or NPCs, neural organoids offer an entire set of cell types of the human organs.

### 3.1 Brain organoid system in regenerative medicine

Brain disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD), traumatic brain injury (TBI), and stroke, along with several other chronic neurodegenerative disorders, are debilitating diseases that have few treatment options. Stem cell

therapy is likely to provide beneficial effects for the indications of these diseases. The current understanding of brain diseases is mainly based on traditional 2D monocultural cells, animal models, and postmortem examination. Because of the inherent species differences between animals and humans and the individual differences among genetic and environmental backgrounds, it remains a challenge to investigate brain development and associated disorders. To establish better models of human brain development, stem-cell-based 3D brain organoids systematically decipher the developmental rules, presenting the 3D architectures and physiology of the brain. These generated brain organoids show robust neuronal subtypes and glial subtypes and functionality to mimic *in vivo* neural connectivity [24, 25]. In comparison with 2D monocultural stem cell cultures, physiologic conditions closer to the human organism are provided by organoids to support cell-cell and cell-matrix interactions. Therefore, as an ideal cell source, brain organoids have great potential for the reconstruction of lost neural circuits. Brain organoids transplantation strategy is expected to become an effective treatment for neurological defects after brain injury (**Figure 1b**).

Recently, in two studies, scientists transplanted hPSC-derived cerebral organoids into mouse cerebral cortex and successfully generated vascularized organoids, which promoted the progressive neuronal differentiation and maturation and increased cell survival [26, 27]. They observed the widespread axonal extension and precise synaptic connectivity outside the graft area; however, the region-specific long projections and synaptogenesis mapping were not reported in the two studies. Previously, reported approaches produced brain organoids with large lumens and tubes, which results in insufficient oxygen and nutrients support in increasing metabolic needs, making them difficult to apply in transplantation therapy [10, 28]. Recently, an optimized culture protocol was developed to efficiently generate small human cerebral organoids, which presents the benefit of alleviating the risk of cell overgrowth and safety concerns after injecting into the mouse medial prefrontal cortex [29]. The transplanted cerebral organoids extended projections to basal brain regions and generated human glutamatergic neurons with mature electrophysiology [29]. Moreover, mice transplanted with cerebral organoids show potentiated auditory startle fear response, indicating that the organoids can be functionally integrated into preexisting host mouse neural circuits via building up bidirectional synaptic connections, which provides crucial therapeutic strategy for neurological diseases [29].

However, owing to the cellular composition of brain, organoids dramatically changes along the time course of the development, it is necessary to demonstrate which stage of the organoids is best suitable for transplantation. To address this limitation, Kitahara et al. transplanted hESC-derived cerebral organoids at 6w or 10w into mouse cerebral cortex and found that 6w-organoids extend more axons along corticospinal tracts but caused graft overgrowth with higher populations of proliferative cells while axonal extensions from 10w-organoids were smaller in number but enhanced after brain injury 1 week [30]. A similar study reported that 55d and 85d-cerebral organoids were transplanted into damaged motor cortex, indicating that 55d-cerebral organoids can be used as a better transplantation donor for traumatic brain injury (TBI) [31]. Cells from the transplanted cerebral organoids have the capability to support region-specific reconstruction of damaged brain cortex, upregulate hippocampal neural connection protein and neurotrophic factor, and improve of damaged motor cortex. It is also reported that cerebral organoids were transplanted at 55 days to explore the feasibility of organoid transplantation in stroke [32]. Cerebral organoids were transplanted at 6 h or 24 h after middle cerebral artery occlusion (MCAO) surgery, resulting in reducing brain infarct volume and improving

neurological motor function. Furthermore, they also observed that the transplanted cerebral organoids were also related with increased neurogenesis, synaptic reconstruction, axonal regeneration and angiogenesis, decreased neural apoptosis, and rescued more survival neurons after stroke [32]. Although a few works with respect to transplanting brain organoid system were reported, it still has promising technologies in the future treatment of central nervous system diseases. Hence, the effects of the developmental organoid stage and host brain environment should be accurately evaluated when developing an organoid-replacement therapy for brain injury.

### 3.2 Retinal organoid in regenerative medicine

Retinal degenerative diseases, such as glaucoma, RP, and Age-Related Macular Degeneration (AMD), usually lead to irreversible blindness. So does the importance of finding a viable treatment. Regardless of the underlying etiology of retinal degeneration, the common endpoint is the loss of photoreceptors and underlying RPE. Cell replacement strategy provides a good solution for the treatment of retinal degeneration. Although plenty of studies have been made to understand the cellular and molecular mechanisms of retinal disorders, our knowledge is still in its infancy, and the immortalized retinal cell lines have not recapitulated the developmental stages of the human native retina. The new methodological advances in inducing hPSCs into human retina tissues have opened new possibilities for basic research on investigating the therapies or treatments in regenerative medicine [18, 33]. The generated retinal organoids closely resemble many aspects of the real human retina, including retina-specific ribbon synapse [34] and physiological-relevant response to light stimuli [35], which empower researchers to explore the pathogenesis of retinal diseases and pursue cell/tissue transplantation for developing novel treatment options. Because retinal organoids contain all the cell types of human retina, it plays a primary role in the field of transplantation therapy. In this section, we focus on single-cell suspensions isolated from retinal organoids and application of retinal organoids sheets transplantation used for cell therapies in regenerative medicine (**Figure 1b**).

#### *3.2.1 Retinal organoids as a cell source for therapeutic transplantation*

During the previous decade, the aborted human fetal tissues and the hPSC-derived retinal progenitors were two cell sources for transplantation. Representative retinal cell replacement clinical trials are transplantation of hPSC-derived RPE for the treatment of retinal diseases, including AMD and Stargardt disease [36–38]. It has been proved that the mature mammalian retina lacks the ability to accept and incorporate stem cells or to promote photoreceptor differentiation. In 2006, stem-cell-derived precursor photoreceptors were first integrated into the outer retinal layer of degenerating retina and had success in improving vision [39, 40]. However, the strong ethical restrictions and limited cell sources remain a challenge in current transplantation therapies. The retinal organoids that contain abundant retinal progenitor cells (RPCs) can act as an ideal cell source transplantation in retinal degenerative diseases. Zou et al. transplanted effectively purifying RPCs with the surface markers (C-Kit+/SSEA4−) into the retinal degeneration models of rats and mice, showing benefits to the improvement of vision and preservation of the retinal structure [41]. The RGCs are the earliest differentiated cells closely associated with glaucoma. But the population of RGCs in retinal organoids is not substantial as they gradually degenerate following long-term culture time. Thus, prolonging the

survival time of RGCs may provide the possibility for RGC replacement therapy. Several approaches have been taken to improve the short life of implanted RGCs and the length of axons, such as adding extrinsic growth factors [20], combining 2D and 3D protocols [42], and co-culturing with Müller glia [43]. In another animal study, by transplanting purified rod photoreceptors isolated from retinal organoids in defective S- and M- cone opsins, $Nrl^{-/-}$ mouse retinas can restore rod-mediated visual function and be incorporated into the host retina with forming synaptic-like structures in close apposition to mouse interneurons [44]. Interestingly, recent studies contradicted the common view that transplanted photoreceptors integrate into the photoreceptor layer of recipients. They demonstrated that the material transfer between donor rod photoreceptors and host photoreceptors leads to the acquisition of proteins originally expressed only by donor cells [45, 46]. Thus, the mechanism of the photoreceptor transplantation demands reinterpretation.

*3.2.2 Retinal organoid sheet transplantation for improving visual function*

A retinal sheet derived from cultured retinal organoids or fetal retina is another approach to preserving the neural circuitries and improving visual function. Cell suspension strategies consist of transplanting purified photoreceptor precursor cells, whereas retinal sheet transplantations engraft retinal organoids containing both photoreceptor cells and inner retinal neurons. The inner neurons located in the transplanted retinal sheet, which serves as a scaffold and nurturing microenvironment, are conductive to outer layer retinal cells in differentiation and maturation, preserving normal lamination structures. It is reported that the retinal sheet graft can produce less immune activation that enhances life span and the survival rate of transplanted cells, providing suitability approach for therapies of late-stage retinal diseases.

Furthermore, several studies have demonstrated that the transplantation of hPSC-derived RPE cells in AMD patients shows promising outcomes in clinical trials, such as improvement in retinal integrity, maintainability in visual acuity, and increase in vision-related quality of life [47]. Currently, the transplantation of early-stage retinal organoid sheets is verified to establish connections more effectively with host retinal degeneration, and these connections show higher survival rate over time. A series of studies have been performed to investigate whether the transplantation of retinal organoid sheets can differentiate, integrate, and improve visual function in animal models with severe retinal degeneration [48–50]. In 2016, Shirai et al. dissected "retinas" from organoids to get transparent and continuous neural retina sheets and transplanted them into two primate models with retinal degeneration. In both monkey and rat, grafted hESC-retina differentiated into a range of retinal cell types, such as photoreceptors. The photoreceptors were proved to have migrated to the outer nuclear layer and the host-graft synaptic connections were established in those animal models [51]. Similar results were achieved in another study of transplanting the sheets dissected from hESC-derived retinal organoids into retinal degenerate rats [48]. In addition, to enhance functional integration of transplanted retinal sheet, a method in which a genetic modification was used to reduce ON-bipolar cells resulted in efficiently restoring RGC light responsiveness in degenerated retina [52]. However, in those studies, the absence of a well-defined RPE monolayer presents a main limiting factor for retinal sheet transplantation. To overcome this limitation, hESC-derived retinal organoids and RPE monolayer were combined using different bio-adhesives to transplant into immunodeficient Royal College of Surgeons (RCS) rats. The co-grafts were observed to reconstruct the severely damaged retina structure

and improve visual function [53]. Those studies demonstrate the clinical feasibility of hPSC-derived retinal organoids sheet transplantation and provide practical tools to optimize transplantation strategies for future clinical applications.

In retinal tissue engineering, biomaterials are utilized to optimize the models of the human retina. A growing number of biomaterials, especially synthetic polymer scaffolds, such as biodegradable polycaprolactone (PCL) and polylactic-coglycolic acid (PLGA), have been widely used. The remarkable properties of defined synthetic polymer substrate are thin and biodegradable, which can be placed into the retinal subretinal space with minimal physical distortion [54]. In terms of the report, transplanting mouse RPCs cultured on biodegradable thin-film PCL scaffolds with varying surface topographies into the retinal subretinal space help newly integrated mRPCs exhibit potential to guide stem cell differentiation toward photoreceptor fate and to help cells localized to the outer nuclear layer [55]. Another study implanted the human retinal organoids, which are seeded on PLGA sheets into both normal and Chronic Ocular Hypertension (OHT) rhesus monkey retinas. They found that despite the need of immunosuppression for dexamethasone after transplantation, survival and differentiation into retinal tissue were successfully improved [56]. Subsequently, the same group proved again that with the support of PLGA sheets, retinal organoids showed active proliferation, migration and projection of axons into the host optic nerve after transplanting into OHT rhesus monkey eyes [57].

## 4. Comments and future challenges

Development of neural organoid techniques has yielded rapid progress in clarifying the mechanisms of human neural development. Organoids display many characteristics of the organs from which they were made, including cellular anatomy and interaction, genetics, and specific tissue functions, advancing our understanding the neuro of biology, developmental science, and regeneration. Some of the limitations and challenges of neural organoids have been addressed, but emerging technologies are still required to be applied in further study. With respect to brain organoids, many points are needed to be improved, such as the maturation of neuronal and glial cells, reliable anatomical organization, long-range axonal projection and synaptic connections, and the precise construction of neuronal circuits. Providing a physiologically relevant microenvironment and the more complex whole-brain organoids to reproduce the developmental events of the human nervous systems may be needed in the future. Retinal organoids serve as an ideal choice for therapeutic transplantation, which still face many challenges as following: low yield, high heterogeneity, degenerative inner cell layers, and cancerogenesis. The next-generation retinal organoids would be anticipated to have an integrated vascular network, mature microglia system, and pigment layer wrapping around as well as the integration of bioengineering technologies. To achieve the goal, several engineering approaches may be useful: (1) engineered biomaterials to investigate cell-cell and cell-matrix interactions; (2) genetic engineering technology to study various aspects of organoids development and performance; (3) organoid-on-a-chip device to create an optimal microenvironment with the purpose of generating organoids with higher physiological relevance. Furthermore, the next generation of organoids probably needs to integrate more bioengineering technologies, aiming to overcome each approach's limitation and provide a superior, synergistic approach for constructing more complex organoids in regenerative and precision medicine.

## Declaration of competing interest

The authors declare no conflicts of interest.

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