The Study of Retinal Organoids: Development, Modeling, and Transplantation

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Abstract

There are several different retinal diseases prominent within the United States including age-related macular degeneration (AMD), diabetic retinopathy, and glaucoma. These three particular retinal diseases affect a total of 12.5 million people in the US alone, showing that retinal diseases are a very prevalent issue that require an improvement in the methodology of treatment. Retinal diseases can cause blindness and drastically decrease quality of life. To better treat these diseases and provide patients with proper care, it is important to maintain a proper understanding of the retina.

It has been found that retinal organoids (ROs) can be produced through the use of stem cells. When perfected, these ROs can provide advanced modeling of the retina, as well as modeling of novel treatments for retinal diseases and allow for better testing. Additionally, ROs can be modified in order to better depict retinal diseases and their microenvironments. They may also be used for retinal transplants as a method of treatment. However, ROs are still being developed and understood. Before the full use and benefits of ROs can be reaped, the differences between ROs and natural retinas must be understood and minimized. Additionally, various issues within the ROs themself must be tackled in order for their uses to become more advanced.

Background

Before delving into retinal organoids, an understanding of the retina, as well as the eye as a whole, is necessary for proper development and advancements within this topic. The retina is a layer of tissue lining the back of the eye that senses light and is able to relay images to the brain through the optic nerve [1]. The optic nerve contains the outcoming ganglion cell axons as well as the incoming blood vessels that vascularize the retinal layers and neurons [2]. There are several layers and cell types within the retina. The outermost cells are the ganglion cells, which extend to form the optic nerve, as mentioned previously [3]. These cells are responsible for sending electrical signals to the brain that determine shape, contrast, and color of objects that are seen [4]. This is also the layer that is affected when a patient suffers from glaucoma, which is caused by a high intraocular pressure being placed upon the optic nerve, disrupting the signals sent to the brain [5]. Another cell type located within the middle layer of interneurons of the retina is the bipolar cell [6]. These cells act as relay messengers by taking electrical signals from photoreceptor cells and sending them to other retinal cells [7]. However, this is only possible through the horizontal cells, which connect the photoreceptor cells to the bipolar cells, increasing visual sharpness by integrating and regulating the input of photoreceptor cells [8].

But what exactly are photoreceptor cells? These are the rods and cones that are responsible for turning light into electrical signals [9]. Rods focus on night vision as well as peripheral vision, while cones focus on color and central vision [10]. The damage or degeneration of rods within the retina will lead to various symptoms of vision loss including decreased night vision, and damage to cones will cause symptoms such as light sensitivity, decreased color perception, and decreased visual clarity [11]. Having damage to this part of the retina will drastically affect the quality of life of patients.

Under the photoreceptor cells is the retinal pigment epithelium. This layer provides nutrition and waste removal for the photoreceptors, and the accumulation of waste here will lead to various diseases such as AMD or Stargardt disease [12]. Lastly, the final layer of the retina is the choroid, which contains the blood vessels that supply oxygen and nutrients to the retina. In this layer, leaky blood vessels can expand, causing wet AMD and diabetic retinopathy [13].

It can be seen that within this small anatomical body part, roughly 1,094 square mm in area, many different problems can arise, all of which can cause vision loss and drastically decrease quality of life [14]. As such, it is important to be able to provide people with the opportunity for repairment from the damages that may occur. Retinal organoids are key to such opportunities.

An organoid is a tissue formed through stem cells that automatically form three-dimensional structures with various cell types [15]. Because organoids are very similar to the organ structures of the human body, retinal organoids are able to provide a spatial and temporal differentiation of the retina and have been induced from human embryonic stem cells, induced pluripotent stem cells (iPSCs), retinal progenitor cells (RPCs), and mesenchymal stem cells [16]. Due to their similarities to the human retina, retinal organoids are useful for the in vitro modeling of treatments for various retinal diseases. Additionally, they also include all of the cell types of the human retina, allowing for tissue slices from cultured organoids to be transplanted into the diseased layers of a retina, allowing for in vivo treatments [17].

Retinal Organoids and the Natural Retina

Though the development of retinal organoids has been quite advanced, it is still not complete. There has been a lack of attention toward retinal ganglion cells (RGCs) and their development as well as their organization with retinal organoids [18]. The axons of RGCs are what form the optic nerve that allows for signals to be passed from the eye to the brain. As such, having underdeveloped RGCs lead to an improper visual connection, hindering eyesight due to an unstable path for electrical signals [19]. Additionally, many retinal degenerative diseases target RGC axons, causing their deterioration [18]. RGCs have limited capabilities for

regeneration as there is an inability to regrow long distance connections [20]. Due to this, it is important for retinal organoids to delve into this layer of the retina, and several studies have been conducted to observe produce the self-organization and differentiation of RGCs within retinal organoids, providing a stepping stone for the improvement of ROs towards a more accurate replica of the natural retina [21]. However, it appears that stable RGCs that do not degenerate after a short time period have yet to be developed. Additionally, retinal induction protocols are inconsistent in their efficiency and it is difficult to produce mature photoreceptors [22].

Through the use of immunostaining and electron microscopy, ribbon synapses in ROs have been identified. However, these synapses appear to be underdeveloped within the outer plexiform synapses of the horizontal and bipolar cells, as well as the photoreceptors [23]. Furthermore, ribbon synapses between various cell types, bipolar cells, amacrine cells, and ganglion cells, have yet to be detected. This may be due to the same reasons as the occurrence of degeneration of RGCs within ROs. There are several other limitations within the development of ROs that distinguish them from the natural retina. As such, these variations must be studied to isolate their causes and allow for standardization and accuracy of the production of ROs. This includes implementing a Good Manufacturing Practice (GMP)-compliant procedure. To achieve a GMP-compliant protocol for the production of ROs, three key factors have been addressed: cell culture automation, appropriate xeno-free conditions, and cell sources for iPSC line generation [24]. A way to implement cell culture automation is through the usage of a bioreactor, which allows for the increase in reproducibility as well as limitations on the risk for contamination [25].

Methods for Retinal Organoid Development

Previously, retinal organoids have been developed in static cultures, producing two dimensional as well as three dimensional structures [22]. However, this introduces limitations in oxygen as well as nutrient diffusion, leading to hindrances in the development of the organoids [26]. Additionally, it is difficult to control cell differentiation, as well as produce cell organization, cell-cell interactions, and cell-matrix interactions [27]. Two dimensional monocultures lack the ability to mimic cellular functions and signaling pathways found in tissues, demonstrating how stem cells must be developed further past this point in order to produce retinal organoids that can be used as proper models and treatments [27]. It has been found that if the proper three dimensional scaffold and biochemical factors are implemented, differentiated pluripotent stem cells (PSCs) will self-organize to form tissue-specific organoids, including retinal organoids [27]. These biochemical factors include small molecules used for cell survival, proliferation, and self-renewal which are all important for the growth of the organoids. Additionally, an important component used for these scaffolds to better develop organoids is matrigel, which provides signaling through the basement membrane ligands [28]. The basement membrane is important for epithelial tissue organization and function [29]. As such, the matrigel supports cell attachment and survival, leading to organoid formation [27].

However, matrigel is not well-defined and does not facilitate the specific extracellular matrix (ECM) cues that are necessary for the development of different tissue types [27]. This makes it difficult to use to obtain specific organoids [30]. These kinds of scaffolds also lead to hindrances in reproducibility and inconsistencies in the growth of organoids [31]. This is due to variabilities that have been found between different batches of matrigel, specifically within its ingredients as well as its mechanical properties [32]. Without being able to standardize the production of matrigel that is commercially available, the growth of organoids will remain inconsistent. As such, various methods for non-matrigel scaffolds have been researched to develop a method that provides proper structuring and biomimetic properties. It has been found that natural polymer-based hydrogels are a preferred material for scaffolds due to their similarities to human ECM in chemical composites and fibrous structures, providing an advantage for cell growth and cell differentiation [30]. These hydrogels are polysaccharides, proteins, and animal-derived mixtures. Polysaccharides, such as gelatin, agarose, alginate, hyaluronic acid (HA), and cellulose, have fast gelation properties that allow them to be well-suited for the development of complex 3D scaffolds that may contain various forms such as microfibers, microspheres membranes, and defined blocks [32]. Polysaccharides also have the advantage of being biodegradable, making them more well-suited for in vivo transplantation of organoids [30]. Additionally, natural proteins including gelatin, fibrin, collagen, and silk protein have been found to interact with cells, as well as provide proper microenvironments, guiding cellular behaviors through componential cues [30]. This is an improvement from matrigel, which lacks the capabilities to deliver ECM cues that facilitate cellular growth and differentiation. Furthermore, a more biocompatible scaffold can be produced through animal-derived mixtures of polysaccharides and proteins, as well as decellularized ECM [33]. These scaffolds provide more catering towards the biocompatibility as well as bioactivity of specific tissue types, allowing for cell survival and growth [30].

While these natural polymer-based hydrogels appear to have various advantages, they do not contain proper mechanical properties and stability for the growth of organoids [34]. However, adding chemical cross-links improves these issues and allows for the scaffold immobilization and effective release of active agents and biomolecules [35]. Adding chemical cross-links also has drawbacks as it increases toxicity to the organoids [36]. As such, self-crosslinking hydrogels made from natural materials may provide a better alternative and can lead to the development of stimuli-responsive hydrogels [37]. The mechanical properties of these hydrogels are not as up to par as the chemical cross-linked hydrogels, but they provide designs that can lead to better organoid formation.

While there have been many studies and developments on the optimization of scaffolds for organoid formation, the growth of such tissues requires more than a static environment. A study conducted by Distefano et. al. developed a method for the betterment of retinal organoid

growth using a rotating-wall vessel (RWV) bioreactor [38]. This bioprocess was used to culture retinal organoids derived from mouse pluripotent stem cells using a matrigel scaffold. Cell aggregates were placed within a rotating cylindrical vessel with a central porous core. This core provided a culture medium and allowed for waste disposal through perfusion. The rotation speed of the vessel was also optimized to maintain cell aggregates in stationary suspension, which was done by balancing out the motion of the medium with the settling of the aggregates, creating a simulated microgravity [38]. By providing this stationary suspension, shear force as well as mechanical interactions with the vessel walls were limited, minimizing tissue damage and producing an optimized environment for vulnerable tissue types, such as the neural retina (NR) [39]. In this study, the growth of retinal organoids was observed within the RWV, as well as in a static suspension culture (SSC) to provide comparisons of this novel method. The NR within the RWV was dissected, while the SSC experiments had both dissected NR and intact NR, SSCd and SSCi. Figure 2 below shows the results of this experiment.

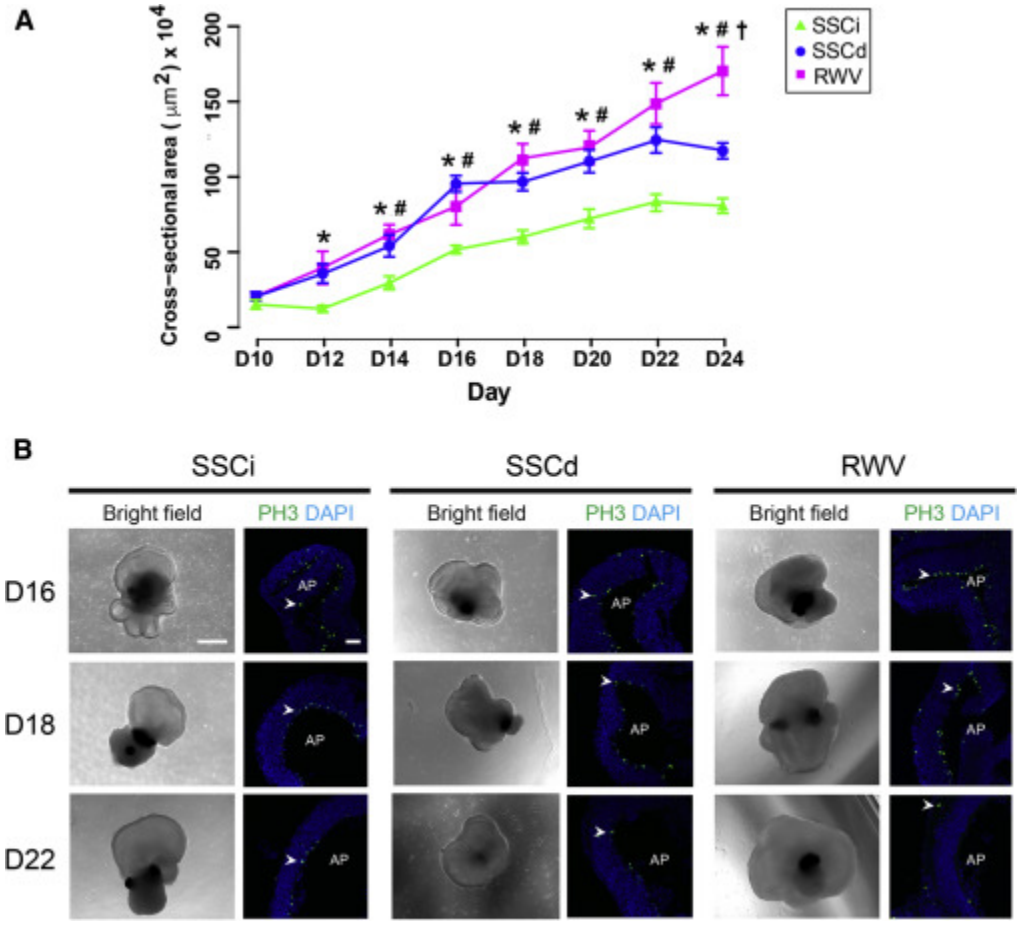

Figure 2 reproduced with permission from Elsevier [39]

A) graph of retinal cross-sectional area over 24 days of retinal organoid in RWV, SSCd, and SSCi

B) Bright field and staining images of ROs in RWV, SSCd, and SSCi

It can be seen in Figure A of Figure 2 that the dissected NR within the RWV maintains a statistically significantly larger cross-sectional area as opposed to the NR within the SSCd and SSCi. The SSCi NR is continuously smaller than both of the other NR types after 12 recorded days of the experiment, while the SSCd NR is similar to the RWV NR up until day 14 of the experiment, and does not see much increase in size after day 16. Figure B of Figure 2 shows the bright field image as well as the stained image of the NR. The staining was done using phospho-histone H3 (PH3) for proliferating cells, and DAPI for nuclei. By day 22 of NR in RWV, it can be seen that there were few proliferating cells, suggesting that the cells had begun differentiating into different retinal cell types.

It has been mentioned previously that the differentiation of RGCs within retinal organoids has been limited. With the method of RWV, RGCs appear to thrive, which may be due to the mass transport of oxygen, nutrients, and metabolic waste [39]. However, the survival of RGCs could not be sustained throughout the later stages of development. Furthermore, Calbindin (CALB), a biomarker for horizontal and amacrine cells, was found at day 15 in the SSCd and RWV cultures in the neuroblastic layer. Two layers of CALB cells were also found in RWV at day 22, SSCd at day 28, and SSCi at day 32, suggesting that the development of horizontal and amacrine cells are accelerated through the dissection and simulated microgravity microenvironment. In addition to this, RWV cultures appear to have greater capabilities in the differentiation and maintenance of cone photoreceptors, especially the S-cones, which were found to be minimally occurring in static cultures. At day 22, the RWV organoids had a high number of S-cone photoreceptors and a high expression of S-opsin, which was observed to be maintained until day 25, unlike both types of static cultures, which even observed a drastic decrease of S-opsin immunostaining [39]. These results indicate that the RWV culture conditions lead to accelerated differentiation of retinal organoids, with organoids in day 22 RWV having greater differentiation of major cell types in comparison with organoids in day 28 SSCd and day 32 SSCi [39].

Overall, it can be seen that implementing the method of RWV for retinal organoid formation has led to a drastic improvement in the cellular differentiation of various cell types in comparison with the former methods of SSC. This may be due to the reduction of shear force placed upon the cell cultures that is obtained by producing the simulated microgravity environment. Additionally, dissecting the NR within the SSC also appears to produce an improvement in the growth of the organoids, and the use of this method within the RWV appears to have an effect upon the improved organoid growth. However, it seems that there is a need for another trial of this experiment to be conducted to observe intact NR within the RWV in order to better understand how much of the increase in the retinal cross-sectional area is due to the

bioreactor and how much is due to the dissection. In other words, which of these two factors is dominating this observed increase?

Retinal Organoids as Models for Retinal Diseases

There have been over 20 studies that have used patient-derived or gene-edited human PSC-derived retinal organoids (ROs) for the purpose of modeling inherited retinal diseases [40]. These include models for retinitis pigmentosa (RP), Leber's congenital amaurosis (LCA)-related RPE65, CEP290, AIPL1, CRX, glaucoma, macular telangiectasia type 2, microphthalmia, retinoblastoma, Stargardt disease, and *RS1*-related X-linked juvenile retinoschisis, demonstrating that ROs provide a wide variety for retinal modeling of various diseases, which can lead to improved methods of treatment [40]. Additionally, a study conducted on SARS-CoV-2 found that the virus can infect retinal cells [41]. Angiotensin-converting enzyme 2 (ACE2) has been found to be the main host cell receptor of which the SARS-CoV-2 S1 protein binds to and allows for entry into the cell [42]. Due to patient reports of nervous tissue as well as eyes being affected by the virus, human induced pluripotent stem cell (HiPSC)-derived ROs and monolayer cultures derived from ROs were grown to observe the potential virus entry into the retina [41]. It was found that both the HiPSC-derived ROs as well as the monolayer cultures express ACE2 on the mrna level, and immunofluorescence staining confirmed ACE2 protein expression within them. Additionally, a SARS-CoV-2 pseudo-virus spike protein was used to observe that the ROs and monolayer cultures were susceptible to infection from the virus [41].

In addition to disease modeling, ROs developed from human iPSCs have been used to observe the effects of moxifloxacin [43]. The antibiotic appeared to produce the same reactions in the ROs as it did in adult mouse retinas, primarily affecting the photoreceptors [43]. This experiment demonstrates how ROs can be properly developed for the usage of pharmacology and drug screening. Additionally, experiments have been conducted taking somatic cells from patient biopsies into iPSCs used for RO development and observing effects of mutations in CEP290, a centrosome-cilia protein [44]. It was found that when derived from patients with Joubert syndrome, the cilia formation was impaired. However, cilia formation remained relatively normal when ROs were developed from patients with Leber congenital amaurosis. This difference in RO development based on the stem cells used from patients demonstrates that there is potential for disease specific and patient specific models to be developed, allowing for more therapeutic and experimental opportunities. These experiments also highlight the use of ROs as models for observing not only the degeneration of retinas due to retinal diseases, but also for observing various diseases and infections not specific to the retina and their effects on this tissue, as well as methods for treatment. Using ROs for modeling allows for a better understanding of diseases and infections, broadening knowledge depth and opening more pathways for patient care and treatment.

Retinal Organoids for Transplantation

In addition to the various modeling uses of ROs, they may also be used for transplantation for the treatment of diseases. For example, patients who suffer from glaucoma will typically also see loss in ganglion cells that may be as a result of mechanical compression, abnormal blood supply, and immune mechanisms [15]. One study looked into the transplantation of retinal progenitor cells (RPCs) in human embryonic stem cell (hESC)-derived ROs into the ganglion cell layer of mice to observe their differentiation in vivo into RGCs [45]. It was found that the transplanted cells expressed Brn3a, an RGC-specific marker, showing that the RPCs could integrate into the host ganglion cell layer and develop into RGCs in vivo. Having this capability could drastically improve retinal degeneration that targets ganglion cells, such as glaucoma, providing patients with a new form of treatment. However, the number of ganglion cells in ROs was found to gradually decrease over time, with their identification lasting for only up to 4 weeks [46]. As such, to study RGCs within organoids, a method must be developed to produce them in larger numbers. It was found that converting three dimensional organoids after the development of the optic vesicle into two dimensional cultures effectively increased the differentiation of ganglion cells, achieving a rate of 90% differentiation [47].

Furthermore, to better the transplantation of RGCs, an appropriate axon length is required [48]. Having an axon that is too short in length will cause issues to arise within the synaptic connections between the ganglion cells and the visual pathway [15]. As such, it is important to study the factors that affect the length of the axons in order to improve RGC replacement therapies. One study has found that the maturity of the organoids from which the RGCs are collected may play a role in their axon length. It was found that RGCs collected from organoid cultures at days 90 through 110 of growth experienced longer neutrites than those collected at younger stages of differentiation, which allows for an easier connection with the brain [49]. The maturity of RGCs can be observed through the RGC markers CD184 and CD171, with the CD171 marker being present in greater amounts in mature RGCs while ganglion cell precursors expressed higher levels of CD184 [50]. Observing the levels of these markers allows for the tracking of the maturity of the RGCs in order to better develop ganglion cells with longer axons for better transplantation.

Conclusion

Due to the similarities in cell types and structures between the retina and ROs, the organoids can be used as an accurate model for the study of various diseases affecting human retinas. They may also be used as models for treatment methods of these diseases, providing a more accurate methodology of in vitro testing. In addition to their modeling capabilities, ROs can also open the field into organ transplantation, further broadening treatment methods and increasing quality of life in people suffering from retinal damage. However, there is still much

work that is necessary to be done in order to perfect ROs for these uses. One obstacle that has been found to be hindering the progress in ROs is the development of RGCs, which are important in establishing proper connections between the retina and the visual pathway. RGCs have been found to either be underdeveloped or degenerate quickly within ROS. As such, various methods for developing ROs have been established, such as using various scaffolding techniques and materials, as well as implementing the usage of RWV bioreactors. Additionally, more specific techniques have been introduced for the development of RGCs within the ROs, including methods for mapping the maturity of the cells for appropriate collection times. However, these methods have still to be perfected before the application of ROs can be implemented into clinical practices and provide better patient care. The study conducted by DiStefano et. al. found that the usage of the RWV bioreactor did statistically increase the growth of retinal organoids. However, this study was conducted using matrigel as the scaffold for the organoids, a material that has been found to lead to inconsistencies and reproducibility in organoid growth. Natural polymer-based self-crosslinking hydrogels have been proposed as an alternative to this material as results have shown it to be favorable. As such, using these hydrogels for the scaffold within the RWV bioreactor may lead to even better results in retinal organoid growth, taking a step closer to perfecting them for usage in disease modeling as well as for transplantation.

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