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Deciphering Retinal Diseases Through the Generation of Three-Dimensional Stem Cell-Derived Organoids: Concise Review

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ABSTRACT

Three-dimensional (3D) retinal organoids, in vitro tissue structures derived from self-organizing cultures of differentiating human embryonic stem cells or induced pluripotent stem cells, could recapitulate some aspects of the cytoarchitectural structure and function of the retina in vivo. 3D retinal organoids display huge potential for the investigation of the pathogenesis of monogenic hereditary eye diseases that are related to the malfunction or degeneration of photoreceptors or retinal ganglion cells by providing an effective in vitro tool with multiple applications. In combination with recent genome editing tools, 3D retinal organoids could also represent a reliable and renewable source of transplantable cells for personalized therapies. In this review, we describe the recent advances in human pluripotent stem cells-derived retinal organoids, determination of their histoarchitecture, complexity, and maturity. We also discuss their application as a means to decipher the pathogenesis of retinal diseases, as well as the main drawbacks and challenges. STEM CELLS *2019;37:1496–1504*

SIGNIFICANCE STATEMENT

Three-dimensional (3D) retinal organoids display huge potential for the investigation of the pathogenesis of monogenic hereditary eye diseases that are related to the malfunction or degeneration of photoreceptors or retinal ganglion cells by providing an effective in vitro tool with multiple applications. In combination with recent genome editing tools, 3D retinal organoids could also represent a reliable and renewable source of transplantable cells for personalized therapies. In this review, we discuss the recent advances in pluripotent stem cell-derived retinal organoids and discuss their application as a means to decipher the pathogenesis of retinal diseases.

INTRODUCTION

The human retina is a highly complex organ that forms the lining of the inner surface of the rear of the eye and represents an integral part of the central nervous system. The retina consists of the neural retina and a nonneural supporting layer of retinal pigment epithelium (RPE). The neural retina is a stratified structure made up of 55 different cell types, including various types of horizontal cells, bipolar cells, numerous types of amacrine cells, Müller glia, retinal ganglion cells (RGCs), and photoreceptors (cones and rods) [1]. Photoreceptors receive and convert light into nerve signals that pass through bipolar cells to the ganglion cells that conduct action potential to the rest of the brain. A significant amount of visual processing arises from the patterns of communication between different cells in the retina. Retinal diseases and disorders that impair vision are often caused by the degeneration or dysfunction of the cells that make up the retina; for example, photoreceptors and RPE are implicated in heterogeneous retinal diseases including inherited retinal dystrophies (i.e., retinitis pigmentosa [RP] and Leber congenital amaurosis [LCA]) and age-related macular degeneration, whereas RGCs are implicated in a range of optic neuropathies such as glaucoma.

Cellular and animal experimental models have been widely used to dissect the mechanisms involved in retinal diseases. Many of the animal models developed closely mimic the gene mutations observed in human patients [2]; however, interspecies differences in eye size, refractive properties, retinal vasculature, and visual photopigmentation must be taken into account when extrapolating findings discovered

in, for example, mouse eyes to human eyes [3]. Mice, as nocturnal animals, have more rods than cones, thereby making the comparison between these two species difficult. Heterologous cellular expression systems, which overexpress mutated genes, are widely used as simple and reproducible alternatives, but the lack of endogenous regulation of the implicated genes hampers this approach [4]. Furthermore, postmortem patient samples remain difficult to obtain and generally represent only the most advanced stage of the disease. Therefore, retinal cells derived from patient-specific stem cell sources may be the appropriate platform to study the pathogenesis of retinal disease.

Human-Induced Pluripotent Stem Cell (hiPSC) Technology and Retinal Differentiation

The deficiencies encountered when assessing retinal disease using animal and cell models may be overcome by generating sophisticated human cellular systems derived from patient cell samples, which display the required genetic diversity. A Nobel-prize winning report regarding the generation of hiPSCs via the reprogramming of somatic cells, by the Yamanaka and Thomson groups in 2007 [5, 6], revolutionized health care and personalized medicine, and enabled the rapid production of disease-specific cellular models.

The multilineage differentiation capacity of hiPSCs offers a model platform to study retinal dystrophies, as their cell fate can be restricted toward RPE, photoreceptors, and/or RGCs [7–10], thereby providing an unlimited cell source to investigate the disease at the molecular, cellular, and functional level.

The first studies describing the production of photoreceptors and RPE cells from hiPSCs under two-dimensional (2D) adherent culture conditions, reported the generation of photoreceptor progenitors, with few cells expressing mature photoreceptor markers [10, 11]. The Takahashi group [10, 11] tried to model RP diseases in vitro by deriving hiPSCs lines from RP patients carrying mutations in the *RP1*, *RP9*, *PERIPHERIN*2, and rhodopsin genes, which affect the photosensitivity and outer segment morphogenesis of rod photoreceptors. Although the protocols followed the processes and used patterning molecules that exist during retinogenesis, the generation of mature photoreceptors remained a difficult challenge. As most retinal dystrophies reflect photoreceptor dysfunction and loss, there existed a clear need to focus research efforts toward the development of new protocols for the efficient differentiation of hiPSCs to achieve greater photoreceptor yields.

This feat was achieved via the construction of 3D differentiation procedures that generated multilayer optic vesicle-like structures comprising multiple retinal cell types and displaying the ability to form retinal laminae [12–15]. Overall, the application of 3D models allowed the generation of tissue more closely resembling some of the native features of neural retina at the microarchitecture level (Fig. 1) when compared with the efforts derived from 2D approaches.

3D Organoids Recapitulate Eye Development to Generate Functional Retinal-Like Structures

Pioneering work from the Sasai group based on mouse embryonic stem cells created the first fully defined 3D retinal organoid structures [16]; they demonstrated optic-cup morphogenesis in cell culture as an intrinsic self-organizing program, involving stepwise and domain-specific regulation of local epithelial properties. During development, retinogenesis begins with the optic vesicle forming as an evagination of the diencephalon. The vesicles are then transformed into two-layer optic cups consisting of the inner neural retina and nonneural RPE. The neural retina continues to form stratified structures made of layers of photoreceptors and other cell types, including horizontal cells, bipolar cells, and amacrine cells. Sasai and colleagues used embryonic bodies (EBs), 3D aggregates that have the capacity to form cells from three embryonic layers [16]. The maintenance of EBs as floating aggregates allowed the spontaneous formation of retinal primordial tissue buds, mimicking the morphological tissue invagination of the neuroectoderm in vivo and resembling the optic vesicle. Further differentiation of the optic vesicles permitted the generation of complex retinal stratified structures in vitro.

Further improvements made by the Sasai group to allow the generation of optic vesicles from hESCs [17] used the extrinsic modulation of several pathways, including Wnt, Sonic Hedgehog, and fibroblast growth factor (FGF) signaling normally required during mammalian development, and used additional extracellular matrix components (Matrigel). Improved protocols using bone morphogenetic protein (BMP), transforming growth factor beta (TGFβ), and Wnt antagonists directed hESCs toward a neurogenic fate with a subsequent optic cup-like structure formation [18]. Other approaches to generate retinal tissue in vitro revealed the different aspects of morphological and functional maturation of retinal organoids (also known as retinospheres, miniretinas, optic cups, optic vesicles, and 3D retinal tissues) [13, 15, 19–22]. We will not enter into more details of each protocol, as that is not the aim of this study; the essential features of each can be found in Table 1.

To represent reliable models of retinal diseases, the 3D retinal organoids have to approximate some or all features of the human retina in vivo. In the next section, we hope to summarize the main achievements related to retinal organoids derived from human pluripotent stem cells (hPSCs), the comparisons made to retinal structures in vivo, and the determination of their complexity and ask whether these structures could represent reliable human models for retinal disease.

3D Retinal Organoids Recapitulate the 3D Histoarchitecture and Functionality of the Human Retina

As previously mentioned, a number of described protocols have permitted the generation of retinal tissue from hPSCs with varying efficiencies allowing us to mimic the early stages of human eye development and to model human retinal disease pathogenesis and repair [15, 17, 19, 21–27] (Tables 1 and 2). These stratified structures display some human-specific features: they are larger in size compared with mouse counterparts and contain different retinal structures, including an outer nuclear layer with photoreceptors (rhodopsin+, recoverin+), an inner nuclear layer with horizontal (calbindin+, calretinin−), bipolar (Chx10+, Pax6−), amacrine (Pax6+, calretinin+), Müller glia cells (CRALBP+), and a RGC layer with RGCs (Brn3+, Pax6+, calretinin+) (Fig. 1). Surprisingly, hPSCs derived retinal tissues resemble the

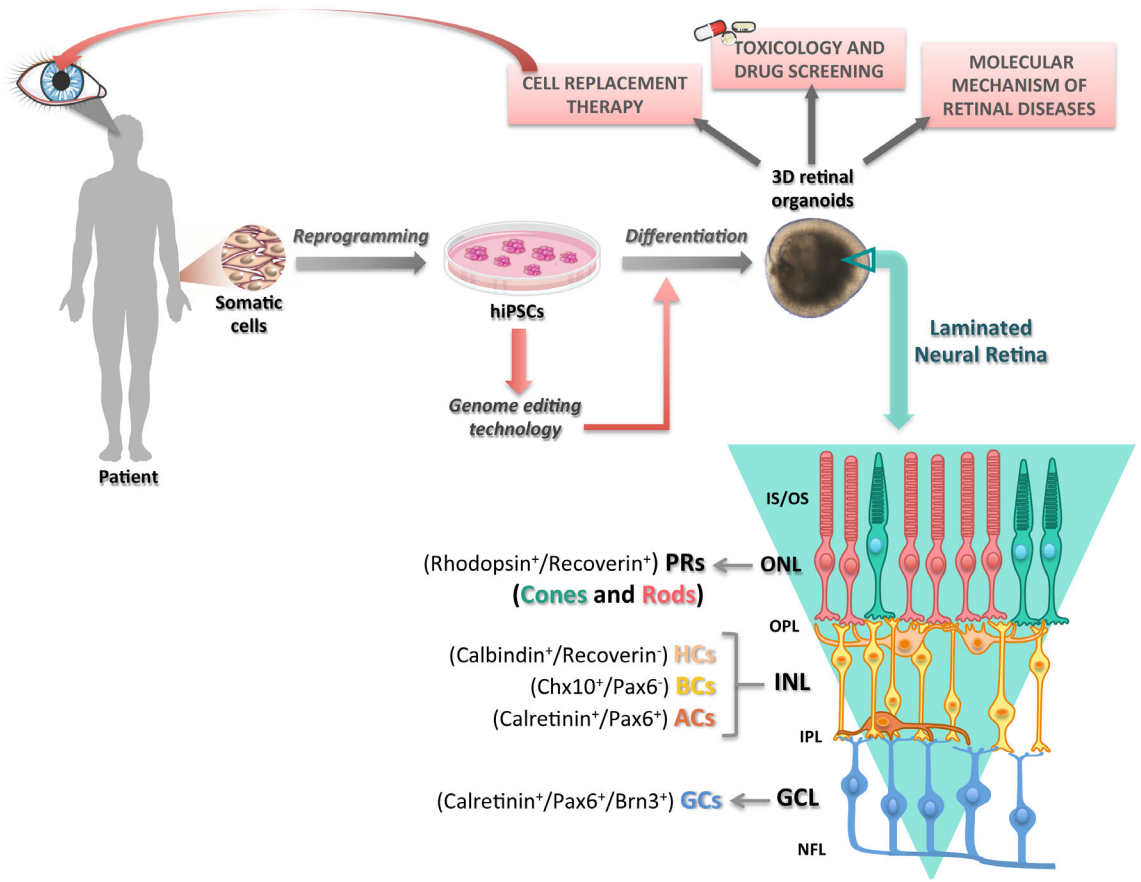

Figure 1. Schematic overview of current hiPSCs disease 3D retinal modeling. Somatic cells from patients are reprogrammed toward human-induced pluripotent stem cells (hiPSCs). Derived 3D retinal organoids from hiPSCs serve as a model for further investigation of disease mechanisms, drug, and toxicological screening as well as for future developments of new therapies in patients. The application of gene-editing technology in patient's hiPSCs could create the gene-corrected 3D organoids as a cell source for transplantation therapy of hereditary retinal dystrophies. Abbreviations: ACs, amacrine cells; BCs, bipolar cells; GCs, ganglion cells; GCL, ganglion cell layer; HCs, horizontal cells; INL, inner nuclear layer; IPL, inner plexiform layer; IS, inner segment; NFL, nerve fiber layer; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, outer segment; PRs, photoreceptors.

in vivo orientation of the retina, presenting an apical to basal polarity [28].

Photoreceptor variety represents a critical aspect of 3D retinal organoids; two types of photoreceptors in the human retina exist—rods and cones—responsible for vision at low or higher light levels, respectively. Their unique morphology or their specific chromophores (rhodopsin for rods and opsin for cones) make it possible distinguish microscopically rods and cones in hPSCs-derived optic cup-like structures.

Zhong et al. [22], Parfitt et al. [29], Wahlin et al. [21], and others [23–25, 30, 31] recently described the examples of organoid photoreceptor outer segments. In their studies, the electron microscopy imaging revealed other ultrastructural features of mature photoreceptors, including an outer limiting membrane, inner segments rich in mitochondria, and basal bodies with connecting cilia displaying a photoreceptor-specific microtubule arrangement. Additional studies have obtained premature forms of stacks of outer segment discs, similar to those observed in the developing human retina [22, 24, 25, 31].

Besides the organizational patterns of retinal cell types in organoids, cell maturity and connectivity within the organoid are required to exploit the full potential of this cell source for preclinical and clinical studies. The detection of synaptic features represents a crucial step in the assessment of photoreceptor functionality in vitro for disease modeling. The mature inner plexiform layer (IPL) contains two types of synapses: ribbon and non-ribbon; non-ribbon synapses are conventional fast electrical synapses whereas the ribbon synapses transmit their signals tonically and in a graded fashion. Ribbon synapses, not unique to the retina, release the excitatory neurotransmitter glutamate and are involved in the transmission of visual information from the photoreceptors through their interconnecting bipolar cells to the ganglion cells (and on to the brain) [32]. To confirm the maturity of 3D retinal organoids, several studies have used an electron microscopy examination of the IPL and outer plexiform layer to detect photoreceptor ribbon synapses [21, 23–25, 31] revealing synapses between different cell types in laminated neural retina.

The most fascinating feature of 3D retinal organoids is the ability for phototransduction—the process in which light is converted into electrical signals. Light is recorded and conducted in the photoreceptor outer segment, which triggers protein cascades, leading to the hyperpolarization of the cell membrane potential at the synapse. The necessary proteins and structures (inner and outer segments of the photoreceptors) have been identified in hiPSCs-derived retinal organoids [21, 22, 24, 25, 31].

Table 1. Generation of 3D retinal organoids from hPSCs

Pluripotent stem cell differentiated in vitro	Method of generation/time (days)	Differentiation factors (pathways)[a]	3D histoarchitecture of human retina	Lamination of NR	Cell types (NR) derived in vitro	Maturation of PR	References
TiPSCs (blood-derived hiPSCs)	Floating EBs → adherent culture of neural clusters → floating culture of optic vesicle-like aggregates (108d)	N2, heparin, B27	3D optic vesicle-like structures with RPCs, RPE, and laminated NR	Yes	PR (rods and cones), GC, HC, AC, and BC	• PR-specific markers • Synapses (chemical and electrical)	[15]
hESCs (Crx:: venus-knock-in)	Floating SFEBq-cultured hESC aggregates (126d)	IWR1e, Y-27632, Matrigel, FBS, SAG, CHIR99021, DAPT, N2	3D optic-cup with RPE and fully stratified NR	Yes	PRs (rods and cones), GC, and interneuron precursors	• PR-specific markers • IS, CC, but not obvious OS	[17]
hESCs (H9-CRXp-GFP)	Floating SFEBq-cultured hESC aggregates (180d)	IWR1e, Y-27632, Matrigel, FBS, SAG, RA, N2	3D optic-cup with fully stratified NR	Yes	PR (rods and cones), GC, HC, AC, BC, and MGCs	• PR-specific markers	[26]
hESC reporter lines (Rx::Venus, Rx:: AcGFP and Crx:: Venus)	Floating SFEBq-cultured hESC aggregates with "induction-reversal culture" method (150d)	d0-d18: Y-27632, BMP4 d18-d24: CHIR99021, SU5402 → RPE induction d24-d150: FBS, RA, taurine → NR induction	3D optic-cup with RPE and fully stratified NR	Yes	PRs, GC, and interneuron precursors	• PR-specific markers	[27]
hiPSCs	Confluent hiPSCs culture (-FGF2) → floating culture of NR-like structures (112d)	N2, FGF2, DAPT	NR-like structures and RPE	Yes	PR (rods and cones), GC, HC, AC, BC, and MGCs	• PR-specific markers • Formation of potential cilia and OS	[19]
hiPSCs	Confluent hiPSCs culture → floating culture of hiPSCs aggregates (7d) → adherent culture of hiPSCs aggregates (21-28d) → floating culture of NR to form retinal cups (190d)	N2, B27, FBS, taurine, RA	3D retinal cups	Yes	PR (rods and cones), GC, HC, AC, BC, and MGCs	• PR-specific markers • OLM, IS, CC, and OS with disc membranes • Phototransduction and photosensitivity	[22]
hPSCs (hESCs and hiPSCs)	Floating SFEBq-cultured hPSCs aggregates to form optic vesicles (d0-d30) → floating culture of NR to form retinal cups (d30-d300)	d0-d8 → vesicle formation: IWR1e, Matrigel, B27 d8-d30 → retinal induction: SAG, FBS, taurine, RA d30-d120 → PR generation: DAPT d120-d300 → PR maturation: -RA	3D retinal cups	Yes	PR (rods and cones), GC, and MGCs	• PR-specific markers • OLM, IS, CC, and OS • Synapses (morphological, chemical, and electrical)	[21]
hPSCs (hESCs and hiPSCs)	Confluent hPSCs culture to form NRVs → floating culture of NRVs to form RCs	W0-W7 → early retinal diff: -FGF2, N2 W4-W20 → late retinal diff: FBS, taurine, RA	3D optic-cup with RPE and fully stratified NR	Yes	PR (rods and cones), GC, HC, AC, BC, and MGCs	• PR-specific markers • IS, OLM, CC, and nascent OS-like structures with disc membranes • Phototransduction • Synapses	[24]

(Table 1 continued on next page.)

Table 1. (*continued*)

Pluripotent stem cell differentiated in vitro	Method of generation/time (days)	Differentiation factors (pathways)[a]	3D histoarchitecture of human retina	Lamination of NR	Cell types (NR) derived in vitro	Maturation of PR	References
hiPSCs	Floating SFEBq-cultured hESC aggregates with "induction-reversal culture" method (Kuwahara et al. [27]) with adaptation to 96-well plates (scalable to toxicology and pharmacology studies) (up to 150d)	Same used in Kuwahara et al. [27]	3D optic-cup with RPE and fully stratified NR	Yes	PR (rods and cones), GC, HC, AC, BC, and MGCs	• PR-specific markers • IS, CC, and OS-like structures with disc membranes • Synapses (morphological, chemical, and electrical) • Phototransduction and photosensitivity	[25]
hPSCs (hESCs and hiPSCs)	Confluent hPSCs culture → floating culture of hPSCs aggregates (7d) → adherent culture of hPSCs aggregates (25-30d) → floating culture of retinal organoids (175-470d)	d0-d6 (floating EBs): N2, BMP4 (d6) d7-d30 (plated EBs on Matrigel): BMP4 (d7-d16), N2 → B27 (d16) d31-d470 (floating retinal organoids): FBS, taurine, RA (until d100)	3D optic-cup with RPE and fully stratified NR	Yes	PR (rods and cones), GC, HC, AC, BC, and MGCs	• PR-specific markers • ONL, OPL, IS, CC, and OS • Synapses (morphological)	[23]

[a]Differentiation factors pathways: IWR1e (Wnt inhibitor); Matrigel (ECM addition); SAG (Hedgehog signaling); CHIR99021 (Wnt agonist GSK3b inhibitor); DAPT (Notch inhibitor), SU5402 (FGFRi).
Abbreviations: AC, amacrine cell; BC, bipolar cell; CC, connecting cilia; d, day; GC, ganglion cell; HC, horizontal cell; IS, inner segment; MGCs, müller glial cells; OLM, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, outer segment; PR, photoreceptor; w, week.

Other than Opsin and Rhodopsin, retinal organoids express several critical proteins involved in rod phototransduction, including the α-subunit of rod transducin (G T1α), the α- and β-subunits of the rod cGMP-phosphodiesterase (PDE6α β), the rod cyclic-nucleotide-gated-channel α-subunit (CNGA1) and β-subunit (CNGB1), and retinal guanylate cyclase 1 (RetGC1) [22]. Furthermore, the observed sensitivity to cGMP indicates that retinal organoids contain parts of the machinery necessary for phototransduction [12, 13]. Additionally, light sensitivity has been demonstrated by different groups via patch recordings [22, 25], although these are comparable only to early light responses such as those observed in the neonatal mouse retina close to the period of eye opening [25].

3D Retinal Tissue as Disease Model

Alongside studies demonstrating that different eye cup-like structures including 3D retinal organoids may provide essential and fundamental insight into developmental and regenerative processes [17, 20, 33], reports of the utility of these human tissues in eye disease modeling are now emerging [9, 29, 30, 33–35] (Table 2). Most eye diseases involve the dysfunction and degeneration of the retina with blindness as the final outcome, as effective treatments do not currently exist. The generation of hiPSCs from patients with retinal diseases and their differentiation to the affected cells as part of 3D retinal organoids could reveal new insights into the underlying pathomechanisms in human disease (Fig. 1).

Encouragingly, a few studies exist that have successfully used different 3D retinal organoids as a means to model retinal degenerative diseases, including RP and LCA that we will now briefly review.

Usher syndrome, a rare hereditary disease that involves early onset deafness and RP, is caused by mutations in one of more than 10 genes; however, the *USH2A* gene encoding the USHERIN protein is the most commonly affected. Tucker et al. [9] used eye cup-like structures derived from EBs form patient hiPSCs carrying a mutation in *USH2A* to model Usher disease in vitro and to determine the pathophysiological mechanism of this mutation. Although these eye cup structures are more 2D structures derived from retinal rosettes and are far away from multilayer 3D organoids, their study discovered the increased expression of GRP78 and GRP94, indicating protein misfolding and subsequent ER stress [36] when comparing in vitro generated retinal tissue and similar structures derived from healthy individuals.

LCA is an inherited retinal dystrophy that causes childhood blindness, with a strong genetic linkage between mutations in the cilia-related gene *CEP290* that causes mis-splicing and premature termination. Photoreceptors, as highly polar neurons, have distinctive inner and outer segments joined by a connecting cilium where *CEP290* is thought to play an important role in protein trafficking. The potential for 3D retinal organoids to identify the fundamental disease mechanisms of *CEP290* was demonstrated by the Parfitt et al. study [29], in which the authors demonstrated defects to cilia in LCA-hiPSCs-derived optic cups, thereby explaining the retinal-specific manifestation and cilia-related disease mechanisms caused by the *CEP290* mutation. The authors successfully rescued the normal phenotype by applying antisense morpholinos that blocked aberrant splicing, restored the

Table 2. 3D retinal organoids as disease models

Method of generation 3D retinal organoids	Disease/mutation	Disease phenotype	Correction/reversion disease phenotype	References
d0-d5: Floating hiPSC-derived EBs with B27, N2, Priomicin, noggin, Dkk1, IGF-1, bFGF d5-d15: Plated hiPSC-derived EBs on Synthemax d15-d21: +DAPT d21-d30: +aFGF d50-d150: Plated on Synthemax with B27 and N2	Usher syndrome (arRP) *USH2A* (Arg4192His CGC>CAC)	Upregulation GRP78 and GRP94 → protein misfolding and subsequent ER stress	No	[9]
Based on protocol used by Nakano et al. [29]	LCA *CEP290* (c.2991+1655A>G homozygous mutation)	Abnormal *CEP290* splicing and cilia defects	Treatment with antisense morpholino to block aberrant splicing and restore expression of full-length CEP290, restoring ciliogenesis, and normal cilia-based protein trafficking	[29]
Based on protocol used by Kuwahara et al. [27]	RP type 11 *PRPF31* (c.1115_1125del11 and c.522_527+10del heterozygous mutations)	Impaired pre-mRNA splicing correlated with ultrastructural, cellular and functional deficiencies: Shorter microvilli and primary cilia, loss of polarity, reduced barrier function and defective phagocytic capacity in RPE and defective primary cilium morphology and features of degeneration and cell stress in PR in patient-specific retinal organoids	*PRPF31* CRISPR/Cas9-correction restore the key celular and functional phenotypes associated with RP type 11	[34]
Based on protocol used by Kuwahara et al. [27]	RP *RPGR* (c.1685_1686delAT, c.2234_2235delGA and c.2403_2404delAG)	Defects in PR in terms of morphology, localization, transcriptional profiling, and electrophysiological activity. Shorted cilium.	*RPGR* CRISPR/Cas9-correction restore PR structure and electrophysiological property, reversed the observed ciliopathy, and restored gene expression.	[30]
Based on protocol used by Phillips et al. [15]	Microphtalmia *VXS2* (R200Q missense mutation that altered the Arg^{200} residue)	Altered expression of developmental signaling molecules that cause growth retardation and preferential differentiation toward an RPE fate, PR maturation delayed and BC genesis absent.	Exogenous expression of wild-type VSX2 early during retinal differentiation partially rescues the disease phenotype: Reduces RPE production and enhances photoreceptor development but not restores BC markers.	[39]
Based on protocol used by Zhong et al. [22]	RP *CRB1* (c.3122T>C p.(Met1041Thr) homozygote missense mutations; 2,983G>T p.(Glu995)[a] and c.1892A>G, p.(Tyr631Cys) mutations; c.2843G>A p.(Cys948Tyr) and c.3122T>C p.(Met1041Thr) missense mutations)	CRB1 patient organoids develop retinal degeneration: Disruptions at the OLM resulting in loss of adhesion between photoreceptors and MGC with misplaced PRs	No	[33]

[a]Differentiation factors pathways: IWR1e (Wnt inhibitor); Matrigel (ECM addition); SAG (Hedgehog signaling); CHIR99021 (Wnt agonist GSK3b inhibitor); DAPT (Notch inhibitor), SU5402 (FGFRi).

Abbreviations: AC, amacrine cell; BC, bipolar cell; CC, connecting cilia; d, day; GC, ganglion cell; HC, horizontal cell; IS, inner segment; MGCs, müller glial cells; OLM, outer limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; OS, outer segment; PR, photoreceptor; w, week.

expression of full-length *CEP290*, and reestablished normal cilia-based protein trafficking [29].

A study by Buskin et al. also observed disrupted cilia morphology associated with progressive degeneration and cellular stress in photoreceptors and RPE cells from retinal organoids, derived from patients with autosomal-dominant RP (mutation in PRPF31 gene) [34]. Using transcriptomic profiling, the authors provided new insights into the molecular pathogenesis of splicing factors in RP and applied CRISPR/Cas9 gene-editing to rescue full protein expression and key cellular phenotypes in RPE and photoreceptors, providing proof of concept for future therapeutic strategies. The authors discovered that mis-splicing of genes implicated in ciliogenesis and cellular adhesion was associated with severe RPE defects that include disrupted apical–basal polarity, reduced trans-epithelial resistance and phagocytic capacity, and decreased cilia length and incidence.

Another study established the value of hiPSCs-derived retinal organoids generated from patients with different frameshift mutations in the *RPGR* gene [30]. Among many genes causing RP, *RPGR* is one of the most prevalent causative genes, accounting for approximately 16% of RP patients [37]. The authors observed significant deficiencies in photoreceptor function, localization, and structure in patient-derived 3D retinal organoids accompanied with shorter cilia. This study also applied CRISPR/Cas9 to correct the *RPGR* mutation to reestablish the correct photoreceptor structure, electrophysiological properties, and cilia function. This study is a clear example of how more sophisticated 3D retinal models are needed to decipher retinal dystrophies.

The works of Phillips et al. [35] and Quinn et al. [33] demonstrated how hiPSCs-derived retinal tissue derived from patients could be applied to study genes involved in retinogenesis. In the first study, the authors modeled microphthalmia caused by mutations in the Visual System Homeobox 2 (*VXS2*) gene by comparing optic vesicles generated from microphthalmia hiPSCs and healthy donor hiPSCs, thereby providing a paradigm for elucidating transcription factors and signaling pathways triggered by this early development associated gene. Mutations in the Crumbs homolog-1 (*CRB1*) gene are linked to retinal dystrophies that exhibit high-phenotypic variability. In the comparative study by Quinn et al. [33], using human retinal organoids derived from hiPSCs and human fetal retina, they compared the onset of CRB protein expression in both tissues showing that fetal CRB complex formation is replicated in hiPSC-derived retina.

Several recent articles have described protocols for the generation of self-forming 3D retinal organoids as a tool to investigate RGCs. The recent article from the Meyer lab [38] established that hiPSCs-derived 3D retinal organoids can serve as a reliable model of RGC development, including their ability to extend lengthy neurites. This 3D model still is not used in modeling optic neuropathies facing the major drawback of poor survival of RGC in organoids in long-term culture. In cases of human glaucoma disease, modeling the 2D protocols for generation of RGC still presents a reliable option for overcoming the problems of long-term survival of RGC, as was shown by the Ahmad group [39].

3D retinal tissue generated from hiPSCs lines carrying mutations in the *OPTINEURIN* (*OPTN*) gene have also been derived [40], and the RGCs observed in stratified tissue have displayed elevated levels of the apoptotic marker Caspase-3, an occurrence observed in many other neurodegenerative diseases.

Overall, these studies demonstrate that relevant human cells can be derived from patients with different retinal diseases as part of 3D retinal organoids, which partially mimic the highly complex organization of the retina. Additional useful applications of genome editing technology in organoids opens a new era of possibility to create transplantation approaches for future autologous therapy by creating gene-corrected, healthy cells derived from patients with monogenic hereditary diseases (Fig. 1).

Current Drawbacks of 3D Retinal Organoids

In this review, we have attempted to briefly describe the state of the art approaches in the fast-moving field of 3D retinal organoids in disease modeling. Although the generation of 3D optic cup-like structures has revolutionized modern ophthalmology, offering a unique and sophisticated in vitro model of the human eye, the current versions of retinal organoids remain relatively distant from the high levels of complexity observed in the mammalian retina in vivo. For example, 3D retinal organoids must include endothelial cells, to create vascularized tissue similar to the retinal tissue in vivo. To take into account the immune system, the 3D retinal organoids need to be cocultured with patient-derived microglial cells to investigate the additional features involved in retinal degeneration such as inflammation. Complementation of the 3D retinal organoid system with a functional layer of RPE will also provide a more physiologically relevant system, given the crucial role of the RPE to photoreceptor biology. Challenges remain, such as how to shorten the timelines associated with the generation of photoreceptor-containing 3D retinal organoids. Current protocols still suffer hiPSCs line-to-line variability [23] and fully maturated retinal cells still are not achieved.

Possibly the greatest challenge in the use of 3D retinal organoids in disease modeling of late-onset retinal diseases is how to reproduce the effect of aging. Despite long culture time, 3D retinal organoids still present immature photoreceptor outer segment morphology, and, independently of the donor age, are comparable to structures found in the late fetal stage. This stage remains distant from the mammalian adult retina, which exhibits a more complex morphology, stratification, and function [41]. Achieving precise developmental timing in vitro is critical to the investigation of late-stage processes, including late-onset RP, whose symptoms appear mainly in the elderly population. Several strategies have already been applied to overcome the limitations of inherently immature cells in the stem cell modeling of other neurodegenerative late-onset diseases. Although prolonged culture produces limited effects [42, 43], approaches such as the addition of stressors in culture medium (e.g., hydrogen peroxide, MG-132, and kanamycin) [44], the overexpression of Progerin [45], or telomere shortening [46] has been used to induce aging-like features in Parkinson disease cellular models. Unfortunately, these approaches have yet to be applied in the case of 3D retinal tissue.

Conclusions and Future Perspectives

Despite the current drawbacks, the rate of development of new methodologies, such as bioreactors [47] and organ-on-a-chip [48], could substantially accelerate the time to generate more mature populations of retinal cells, as well as to mimic human ocular

compartments, suggesting that we will soon be witnesses to the generation of more sophisticated 3D retinal systems in vitro. At the same time, 3D retinal organoids offer a potential tool to either sort out the photoreceptor precursors (e.g., using CD73+) [49] or directly use a sheet of retinal organoids for transplantation [50], the approaches already used for transplantation in animal models of photoreceptor degeneration. Despite current limits, hiPSCs-derived retinal organoids could give new opportunities for its application in deciphering disease mechanisms, cell replacement therapy, toxicology, and drug screening.

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Author Contributions

A.A.C.: writing the manuscript, final approval, graphical design; F.J.R.J., P.J.: final approval; S.E.: concept and design, writing the manuscript, final approval.

Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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