Chapter

Retinal Organoids over the Decade

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Abstract

Retinal organoids (ROs) are 3D tissue structures derived from embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) in vitro, which characterize the structure and function of retina to a certain extent. Since 2011, mouse and human retinal organoids have been available, opening up new avenues for retinal development, disease and regeneration research. Over the decade, great progress has been made in the development of retinal organoids, which is reflected in the improvement of differentiation efficiency and development degree. At the same time, retinal organoids also show broad application prospects, which are widely used in the construction of disease models. On this basis, the mechanism of disease, drug screening and retinal regeneration therapy have been explored. Although retinal organoids have a bright future, the deficiency of their structure and function, the limitations of differentiation and culture, and the difference compared with embryonic retina still remain to be solved.

Keywords: retinal organoids, retinal differentiation, disease models, retinal degenerative diseases, transplant

1. Introduction

Located in the back of eyeball, the retina is a soft and transparent membrane attached to the inner surface of the choroid and forms part of the central nervous system. The retina can sense light stimuli, convert the light signals it receives into electrical signals, and then transmit them to the cerebral cortex through the optic nerve to form vision [1]. The retina is mainly composed of pigment epithelial cells, photoreceptor cells, bipolar cells, horizontal cells, amacrine cells, ganglion cells, and Müller glial cells [2]. Different neuron types form different layers, and the orderly arranged nuclei and synaptic regions are alternately arranged, forming a complex and orderly layered structure of the retina [3].

Our early research on the retina, derived only from human fetal retinal tissue [4, 5], faced significant challenges due to access difficulties and ethical issues [6]. Beyond that, most of what we know about the retina comes from studying animal retinas, but human and animal retinas differ in composition and function. For example, most mammals have only two types of cone photoreceptors that express S-opsin or M-opsin [7, 8], while humans have a third type that expresses L-opsin [9]; mice, the main subjects of our study, have a higher proportion of rods than humans [10], whose vision is determined by the density of cones in the macula and fovea [11, 12]. Therefore, it is of great significance to develop appropriate human retinal models to supplement animal models.

The establishment of human embryonic stem cell (ESC) lines [13] and the emergence of induced pluripotent stem cell (iPSC) technology [14] have turned our attention to cell research. Early 2D differentiation protocols used exogenous signaling molecules, Wnt antagonist DKK1 and bone morphogenetic protein (BMP) antagonist Noggin, to guide pluripotent stem cells to an anterior neural fate [15–17] and to differentiate into various types of retinal cells, including retinal pigment epithelial (RPE) cells, photoreceptors, and ganglion cells [18–25]. However, 2D differentiation is far from interpreting retinal development in vivo. Retinal development and maturation are regulated by a series of interacting signal networks, such as factors secreted by RPE that promote photoreceptor maturation. Early retinal differentiation produced a single cell type [19–22] and lacked the necessary interaction between cells. Therefore, we still need to find a more perfect model of human retina.

This breakthrough was achieved by constructing a 3D differentiation procedure. Through 3D differentiation, we can obtain retinal organoids that are highly reducible to the development process and complex structure of the retina, which we vividly call it "retina in a dish." In this chapter, we review the development of retinal organoids and show their application in today's life science research.

2. Overview of retinal organoids

In 2011, Sassi's team used mouse embryonic stem cells (mESCs) to construct the first true retinal organoid through a 3D differentiation procedure [26]. In the following year, human retinal organoids were created [27], which is of epoch-making significance, meaning that human research on retinal development and retinal diseases has entered a new stage, and retinal organoids also provide a new and most potential tool for the treatment of retinal degeneration diseases.

During neurogenesis in vertebrates, the development of the retina can be roughly divided into two stages, the appearance of the optic cup structure and the orderly differentiation of seven types of retinal cells. In the first stage, the forebrain splits to form two secondary brain vesicles: telencephalon and diencephalon. In the diencephalon, eye field region first bulges outward to form the optic vesicle, and the distal vesicle invaginates to form the double-layer optic cup, which further develops into the outer retinal pigment epithelium and the inner neural retina (NR) (**Figure 1a**) [28–32]. In the second stage, the inner pluripotent retinal progenitor cells (RPCs) sequentially differentiate into retinal ganglion cells (RGCs), cone photoreceptors, horizontal cells, amacrine cells, rod photoreceptors, bipolar cells and Müller glia cells (**Figure 1b**) [33]. The cone and rod are connected to the retinal pigment epithelium and together form the outer nuclear layer (ONL). After extending to the outer plexiform layer (OPL), they form synapses with bipolar cells and horizontal cells in the inner nuclear layer (INL). On the other side of the inner nuclear layer, bipolar cells, amacrine cells, and ganglion cells form the synaptic networks of the inner plexiform layer (IPL). Müller glial cells span the whole layer of the retina, from the retinal pigment epithelium to the ganglion cell layer (GCL) (**Figure 1c**) [34, 35].

Retinal development in vivo is regulated by a series of transcription factors, signal transduction factors and cell surface factors. In vitro, differentiation of retinal organoids is also a programmed process that mimics development in vivo by adding various signaling molecules in stages. First, stem cells proliferate and aggregate (**Figure 2A**), inducing the formation of embryoid body (EB) (**Figure 2B**) and neuroepithelium (**Figure 2C**), which appear as translucent bright rings under a microscope (**Figure 2D**). And then,

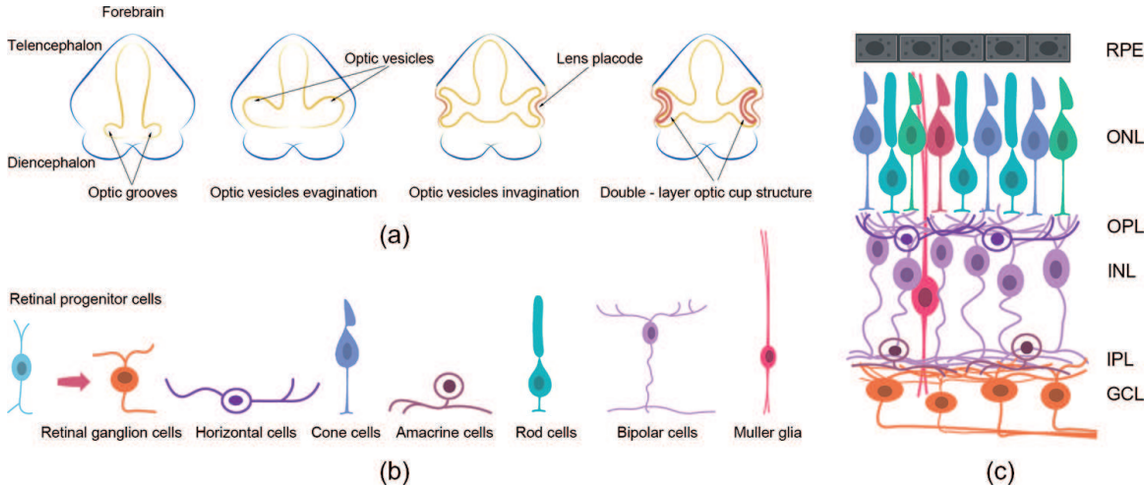

Figure 1.
Overview of retina. (a) the first stage of retinal development: The formation of double—Layer optic cup structure. (b) the second stage of retinal development: Retinal progenitor cells (RPCs) differentiate into seven types of retinal cells. (c) Structure of the retina.

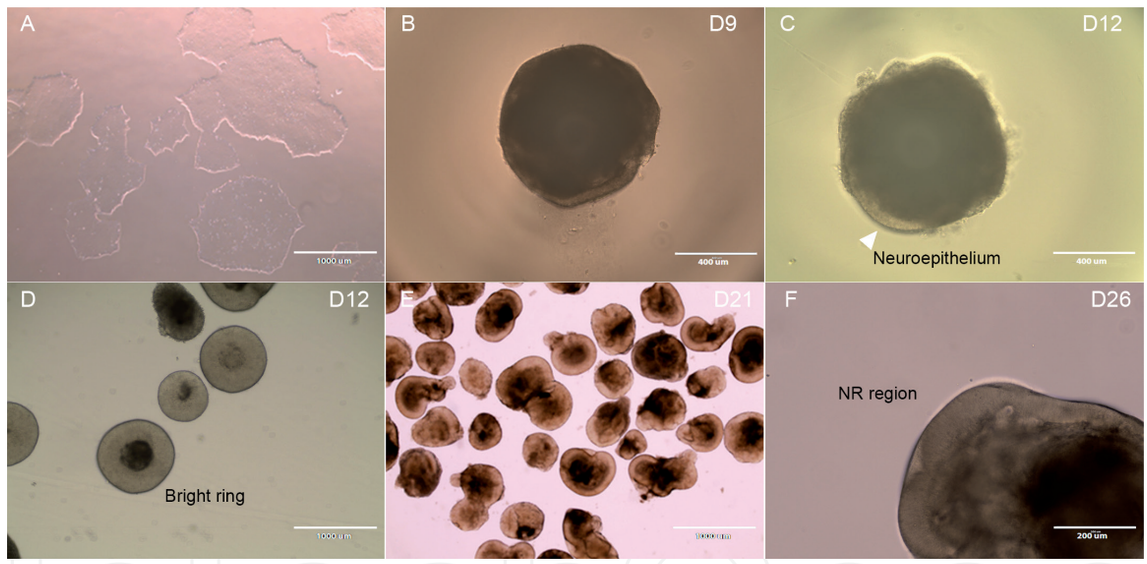

Figure 2.
The differentiation of retinal organoids. (A) Growing human embryonic stem cells (H9). (B) Embryoid bodies (EB) at day 9 of differentiation. (C) Neuroepithelium appear at day 12. (D) Neuroepithelium appear as translucent bright rings at day 12. (E) Optic vesicle/cup at day 21. (F) Neural retinal (NR) region at day 26. Scale bars: 1000 μm (A, D, and E); 400 μm (B and C); 200 μm (F). All photos are provided by Dr. Ze-Hua Xu.

they develop into optic vesicles (**Figure 2E**), followed by neuroretinas (**Figure 2F**), which in turn differentiate into seven types of retinal cells. The sequence of retinal cell types is consistent with in vivo development [36]. After differentiation, the cells undergo spontaneous nuclear migration, forming pinnacles and finally arranged into layered structures, in which the ganglion cells are located in the inner layer of the retinal organoid and the photoreceptors are located in the outer layer of the retinal organoid [26, 27]. Since RPE is usually a mass of cells not adjacent to the neuroretina and is not derived from the floating culture of optic vesicles, we do not consider it to be part of the retinoid organoid in this paper. With the continuous development of differentiation technology, photoreceptors in organoids become more and more mature, which is manifested by the appearance of outer segments and photosensitivity [37, 38].

3. Differentiation of retinal organoids

The development of retinal organoid technology is the result of continuous attempts and innovations by a large number of researchers. Here, we try to review the progress of retinal organoids differentiation in the past ten years (**Figure 3**).

3.1 Diversity of differentiation methods

There are various differentiation methods for retinal organoids, but in terms of differentiation steps, there are mainly two differentiation schemes (**Figure 4**). The first is a classic 3D differentiation protocol from Sassi's team [26, 27]. The stem cells were dissociated and reassembled in a serum-free and low-growth factor medium (SFEBq culture, or serum-free culture of embryoid body-like aggregates with quick aggregation), and forced to form an embryoid body (EB) in a 96-well V-shaped plate.

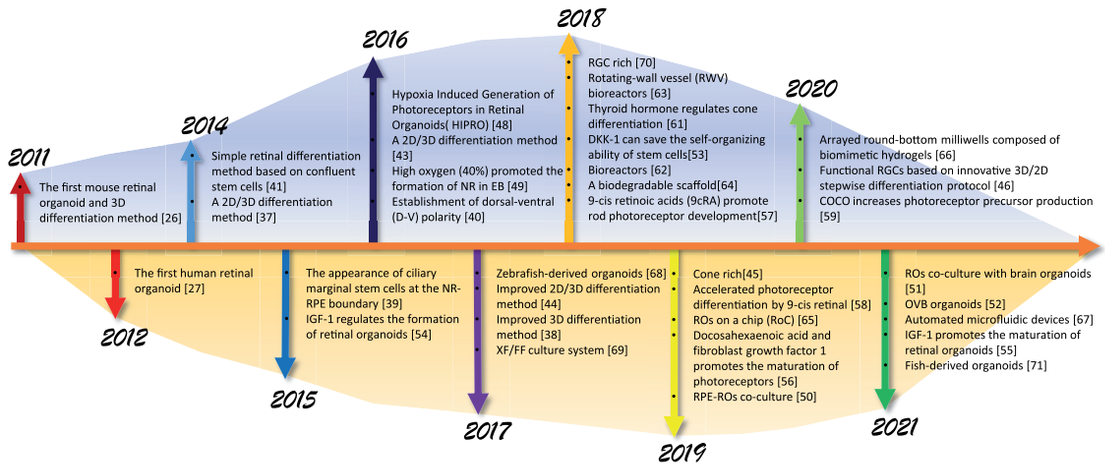

Figure 3.
Progress in retinal organoid differentiation over the decade.

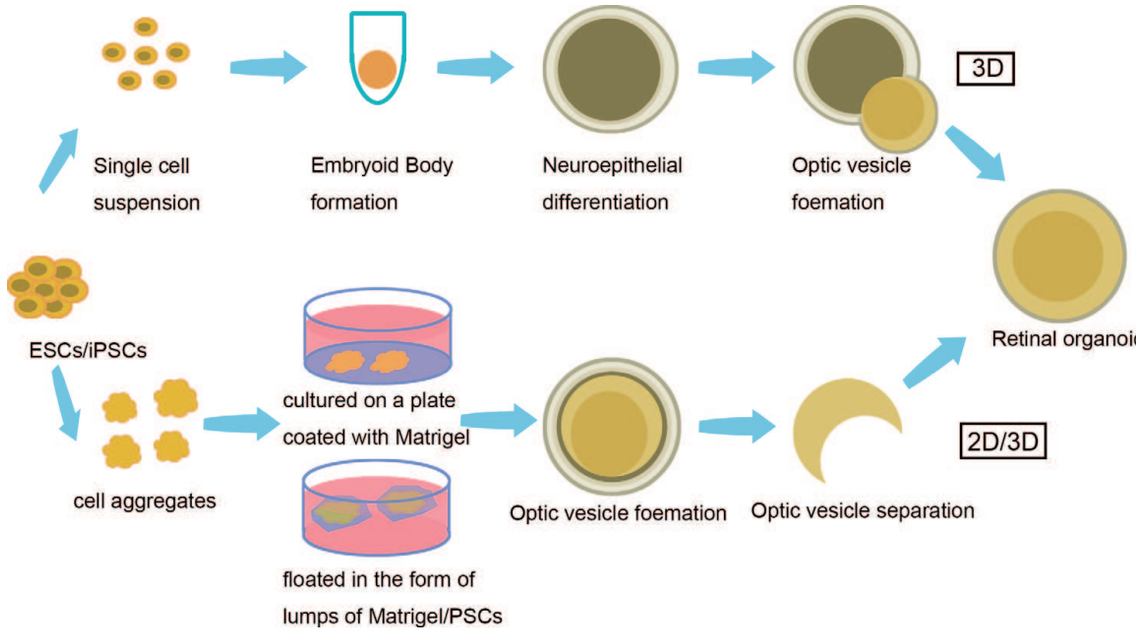

Figure 4.
Two main methods of retinal organoid differentiation.

They were then stimulated by the addition of Matrigel to differentiate into neuroepithelial cells and subsequently into retinal progenitor cells and double-layer optic cup structures [27]. The cells were in suspension culture during the whole process of differentiation, and the formation of optic cups and the differentiation of neuroretina were self-organized [27]. 3D differentiation protocol is complicated in the early stage of differentiation, but it has a higher degree of reduction in the retinal development process, including the occurrence of optic cups invagination, the appearance of ciliary marginal stem cells at the NR-RPE boundary [39], and the establishment of dorsal-ventral (D-V) polarity [40].

The second differentiation method combines 2D culture and 3D culture (2D/3D) [37, 41–46], and the difference is mainly reflected in the early stage of neural induction. It has been reported that pluripotent stem cells can differentiate into the retina even when they are simply fused together [41, 42]. In this differentiation scheme, the stem cells were divided into small pieces by enzymatic hydrolysis [37] or mechanical methods [41, 43, 45] to form aggregates. The aggregates were cultured on a plate coated with Matrigel or floated in medium in the form of lumps of Matrigel/PSCs [43, 45]. After it differentiated into neuroepithelium and optic vesicles, the latter were separated for suspension culture and further differentiated into retinal organoids. This approach bypasses EB formation stage and induces optic vesicle formation by endogenous production of inducer molecules from aggregated cells, avoiding the aggregation step of SFEBq method and the need of Wnt/BMP antagonist [47]. These studies suggest that cell-cell and cell-extracellular matrix interactions are key to inducing retinal organoids differentiation in the early stage of stem cell differentiation.

With the improvement of differentiation methods, the structure of retinal organoids has been improved. Photoreceptors can reach advanced maturity, characterized by the formation of the inner and outer segments and connecting cilia of photoreceptors, the appearance of photosensitivity [37, 44], the expression of photoreceptor neurotransmitters, and the formation of synaptic bands [38, 44]. By adjusting the differentiation method, we can also change the proportion of cells in organoids, such as retinal organoids rich in cones or RGCs [45, 46], which is good for cell transplantation. Oxygen is also an important factor in regulating the differentiation of retinal organoids, and hypoxic conditions (5%) effectively produce vesicles and cups as well as more mature neuroretinas [48]. Another study showed that high oxygen (40%) promoted the formation of NR in EB, as well as the generation, migration and maturation of retinal ganglion cells during metaphase differentiation [49]. The co-culture of RPE with retinal organoids promoted the differentiation of photoreceptors [50], while the co-culture with brain organoids promoted the axon extension of RGCs [51]. More encouragingly, researchers have differentiated human brain organoids with bilaterally symmetric vesicles [52].

3.2 Modulation of signaling molecules

Retinal development requires the regulation of a series of signaling molecules. Similarly, by adding different signaling molecules, retinal organoids differentiation can be regulated in vitro. Dickkopf-related protein 1 (DKK-1), a Wnt signaling pathway antagonist, salvages the self-organizing ability of stem cells to differentiate into retinal progenitor cells [53]. Insulin-like growth factor 1 (IGF-1) regulates the formation of retinal organoids and promotes the formation of the correct retinal lamellar structure by various retinal cells [54, 55]. In the absence of IGF-1, retinal lamination

was absent at the early stage of differentiation, while photoreceptors decreased and retinal ganglion cells increased at the late stage of differentiation [55]. Addition of docosahexaenoic acid and fibroblast growth factor 1 can specifically promote the maturation of photoreceptors including cones [56]. Replacement of widely used all-trans retinoic acid with 9-cis-retinoic acid in culture medium promoted the expression of rod photoreceptors rhodopsin and the maturation of mitochondrial morphology [57, 58]. COCO protein can block BMP/TGFβ/Wnt signaling pathway, enhance photoreceptor precursors, and promote s-cone differentiation and inner segment protuberances formation [59, 60]. During retinal development, s-cone appear first, followed by L/M-cones. This time transition from the designation of the s-cone to the production of the L/M-cone is controlled by thyroid hormone (TH) signaling [61].

3.3 Combination of organoid technology and tissue engineering technology

There is also a lot of innovative research that combines retinal organoid technology with emerging materials technology. The use of bioreactors improved retinal stratification and increased the production of photoreceptors with cilia and new outer segments [62]. In static culture, the development of retinal organoids may be limited by oxygen and nutrient diffusion, and rotating-wall vessel (RWV) bioreactors can accelerate and improve the growth and differentiation of retinal organoids [63]. The spherical structure of retinal organoids limits its interaction with host RPE and the remaining neuroretinas during transplantation. In order to create a planar retinal organoid, a biodegradable scaffold was developed that mimics the extracellular matrix of neuroretinas [64]. Retina-on-a-chip is a new microphysiological model of the human retina that integrates seven different basic retinal cell types and provides vascular-like perfusion to retinal organoids [65]. Arrayed bottom-lined micropores composed of bionic hydrogels, facilitated rapid retinoid tissue formation from mESCs aggregates in an efficient and routine manner [66]. Automated microfluidic devices with significantly reduced shear stress can maintain the long-term survival of retinal organoids [67]. For details of some other differentiation improvements [68–71], please refer to **Figure 3**.

4. Applications of retinal organoids

As a three-dimensional multicellular structure formed by self-organization in vitro, retinal organoids can reproduce the development process of retina in vivo to some extent, and can be used to summarize some structural and functional characteristics of human retina. Meanwhile, they are the most promising tools for retinal disease research (**Figure 5**).

4.1 Retinal organoids as disease models

The reprogramming technique enables iPSCs-derived retinal organoids to retain the patient's genetic characteristics, allowing us to study a variety of retinal diseases in detail in a dish. To date, retinitis pigmentosa (RP), Laber congenital amaurosis (LCA), retinoblastoma (RB) and some other retinal diseases (**Table 1**) have been reproduced in dishes using retinal organoid technology [47, 98].

RP is a relatively common hereditary retinal disorder characterized by night blindness and progressive loss of visual field [99]. LCA, the main disease leading to

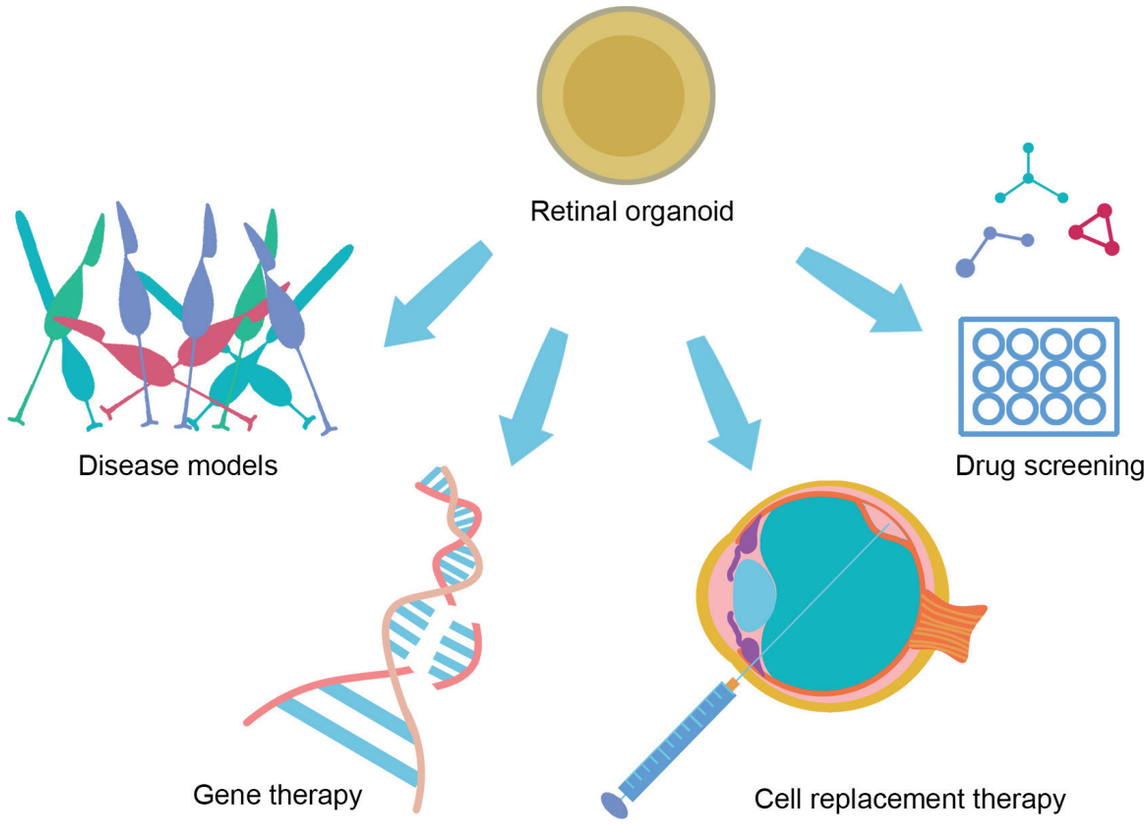

Figure 5.
Applications of retinal organoids.

Disease models	Genetic mutations	Phenotypes of retinal organoids	Reference
RP	Rp11(PRPF31 gene mutation)	Gradually degenerating photoreceptors; disrupted cilia morphology	[72]
RP	RPGR gene frameshift mutation	Photoreceptors have significant defects in morphology, localization, transcription profile and electrophysiological activity; shorted cilium was found in patient retinal organoids	[73]
RP	RP17 structural variants	Increased GDPD1 expression may lead to dysregulation of lipid metabolism, thus affecting photoreceptor function	[74]
RP	USH2A gene mutation	Aberrant organoids polarization, defective neuroepithelium, and abnormal RPCs and photoceptors differentiation	[75]
RP	Crb1 gene mutation	Small but frequent disruptions of CRB complex members at the outer limiting membrane	[76]
RP	PDE6B gene mutation	Increased cGMP levels	[77]
RP	TRNT1 gene mutation	Reduced levels of full-length TRNT1 protein and expression of a truncated smaller protein; autophagy was defective, with abnormal accumulation of LC3-II and elevated oxidative stress levels	[78]

Disease models	Genetic mutations	Phenotypes of retinal organoids	Reference
RP	USH2A gene mutation	Post-developmental photoreceptor degeneration	[79]
RP	REEP6 gene mutation	The expression of a retina-specific isoform REEP6.1 changes destabilize the protein	[80]
XLRP	RP2 gene mutation	Rod cell death peaked at day 150 and the outer nuclear layer thinned at day 180	[81]
LCA	CRX gene mutation	Defective photoreceptor maturation with diminished expression of visual opsins	[82]
LCA	RPE65 gene mutation	Lower expression of RPE65, but similar phagocytic activity and VEGF secretion level	[83]
LCA	AIPL1 gene mutation	Patient-derived organoids maintained retinal cell cytoarchitecture despite significantly reduced levels of AIPL1	[84]
LCA	CEP290 gene mutation	Cilia defects were evident in photoreceptors	[85]
LCA	CEP290 gene mutation	A high level of aberrant splicing and cilia defects	[86]
LCA	CRX gene mutation	Immature and dysfunctional photoreceptor cells	[87]
LCA	CEP290 gene mutation	CEP290 aberrant splicing	[88]
RB	RB1 double allele deletion	Homozygous deletion of RB1 did not affect the maturation and proliferation statuses of human iPSCs (no ROs phenotype)	[89]
RB	RB1 double allele mutation or deletion	Rb originated from ARR3-positive maturing cone precursors during development; tumorigenesis in retinal organoids	[90]
RB	RB1 germline mutation	Retinoblastomas formed from retinal organoids have molecular, cellular and genomic features indistinguishable from human retinoblastomas	[91]
S-cone syndrome	NRL loss	Two distinct populations of s-opsin expressing photoreceptors emerge; one population more representative of typical cones, and the other of rod/cone intermediates	[92]
Rod-cone dystrophy	CRB1 gene mutation	A novel CRB1 transcript displaying skipping of exon 6	[93]
Macular telangiectasia type 2	Normal organoids were treated with deoxy	Dead photoreceptors	[94]
Microphthalmia	VSX2(R200Q) gene mutation	Bipolar cells are absent; delayed photoreceptor maturation	[95]
Stargardt disease	ABCA4 gene mutation	ABCA4 splicing defect	[96]
X-linked juvenile cleft retina (XLRS)	RS1 gene mutation	Retinal splitting, defective retinoschisin production, outer-segment defects, abnormal paxillin turnover, and impaired ER-Golgi transportation	[97]

Table 1.
Retinal organoids as disease models.

congenital blindness in infants, accounts for more than 5% of hereditary retinopathy, with complete loss of binocular cone and rod function within 1 year after birth [100]. Both diseases have been reported to be associated with multiple pathogenic genes. By differentiating different genetically-mutated stem cell lines into retinal organoids [101], we can observe their disease phenotypes in dishes, including photoreceptor degeneration, ciliary morphology disorder, and various functional impairment at molecular levels. Retinoblastoma is the most common intraocular malignancy in children [102]. The main cause of retinoblastoma is the loss of RB1 gene expression [103]. RB1 gene is a tumor suppressor gene, but the mechanism of RB1 deletion leading to retinal cancer is not clear, one of the key questions is the origin of RB cancer. By constructing RB models based on retinal organoids [104], we successfully observed tumorigenesis in retinal organoids and demonstrated that RB originates from ARR3 positive precursors of mature cones during development [90]. Other disease models, such as s-cone syndrome, rod-cone dystrophy, Macular telangiectasia type 2, microphthalmia, Stargardt disease, X-linked juvenile cleft retina, have also contributed to our understanding of retinal diseases.

4.2 Retinal organoids as tools for therapeutic research

4.2.1 Gene therapy

Identification of pathogenic genes promotes the generation of animal models and elucidates the physiological functions of gene products to a certain extent, thus promoting the development of gene therapy. So far, most research has focused on saving retinal organoid disease phenotypes through gene editing of patient-specific induced pluripotent stem cells [72, 73, 87, 97, 105, 106]. However, this strategy cannot be applied to patients. Adeno-associated virus (AAV) show great promise as a gene therapy vector for a wide range of retinal diseases. For example, AAV-mediated gene augmentation has successfully treated LCA caused by RPE65 mutations [107]. AAV-mediated gene therapy based on retinal organoids has also shown promising results in the laboratory [81, 82, 108]. In addition, gene therapies such as antisense morpholino and antisense oligonucleotides (AONs) have also been reported (**Table 2**).

4.2.2 Cell replacement therapy

Hereditary retinal degenerative diseases such as RP, Stargardt's disease and LCA are the leading cause of incurable blindness. The vision loss associated with these diseases is caused by the death of photoreceptors in the retina. Existing treatments, including neuroprotection and gene therapy, require the presence of endogenous photoreceptors. In addition, due to the complex mechanism of retinal degeneration diseases, especially RP, it has been found that there are multiple genes with multiple mutation modes, and treatment methods focusing on a single mutation are extremely difficult technically and economically. Thus, transplant-based photoreceptor cell replacement becomes an attractive therapeutic strategy for restoring visual function and, if successful, could be applied to a wide range of retinal degenerative diseases.

Research on retinal cell transplantation dates back to 2006 [109]. Mice were able to effectively integrate rod photoreceptor precursor cells isolated from juvenile mice retinas into the ONL. These cells can further differentiate in the host retina and exhibit morphological characteristics typical of mature photoreceptors, such as inner and outer segments, while expressing molecules necessary for light transduction,

ROs	Gene therapy	Result	Reference
CEP290-LCA-Optic Cups	Antisense morpholino	Effectively blocked aberrant splicing and restored expression of full-length CEP290, restoring normal cilia-based protein trafficking	[86]
RP11 (PRPF31)-RP-ROs	CRISPR/Cas9-mediated gene correction	Rescued protein expression and key cellular phenotypes in RPE and photoreceptors	[72]
RPGR-RP-ROs	CRISPR/Cas9-mediated gene correction	Rescued photoreceptor structure and electrophysiological property, reversed the observed ciliopathy	[73]
CEP290-LCA10-ROs	Antisense oligonucleotides	Restored wild-type CEP290 mRNA and protein expression levels	[88]
CEP290-Rd16-mROs	AAV-mediated CEP290 fragment	Improved cilia phenotype	[108]
RS1-XLRS-ROs	CRISPR/Cas9-mediated base-editing	Normalized the splitting phenotype, outer-segment defects, paxillin dynamics, ciliary marker expression, and transcriptome profiles	[97]
RP2KO-XLRP-ROs	AAV-mediated gene augmentation	Rescued the degeneration phenotype of the RP2 KO organoids, to prevent ONL thinning and restore rhodopsin expression	[81]
ABCA4-Stargardt-ROs	Antisense oligonucleotides(AONs)	Saved the splicing defect	[96]
G56R-ADRP-ROs	CRISPR/Cas9 mediated gene knockout	ROs differentiation and NR2E3 expression were normal	[106]
CRX-LCA-ROs	AAV-mediated CRX gene augmentation therapy	Partially restored photoreceptor phenotype and expression of phototransduction-related genes; Reduced the loss of opsin expression	[82]
CLN3-RP-ROs	Gene correction	Restored CLN3 mRNA and protein expression and prevented SCMAS and inner segment vacuolization	[105]
CRX-LCA7-ROs	CRISPR/Cas9-based gene knockout	Rescued the photoreceptor phenotypes in organoids	[87]

Table 2.
Gene therapy based on retinal organoids.

forming synaptic connections with downstream cells, generating light responses and promoting visual function [109–115]. These results demonstrate the feasibility of photoreceptor transplantation as a therapeutic strategy for restoring visual acuity after retinal degeneration.

However, this cannot be applied to the treatment of retinal diseases in humans. There are ethical challenges to primary photoreceptors transplantation, but stem cell-based photoreceptors can avoid this problem. It has been shown that photoreceptor cells derived from stem cells can be integrated into mouse retinas, restoring the animal's response to light [116, 117]. This is far from enough, until the appearance of retinal organoids, retinal cell transplantation and clinical transformation have made a breakthrough.

Transplantation of retinal organoids, mainly photoreceptors, is also a process of constant exploration [118]. The safety and effectiveness of transplantation, the enrichment and purification of transplanted cells, the effects of retinal organoids at

different stages of development and host retinas with different degrees of degeneration on the efficiency of transplantation, and the evaluation of cell integration and function after transplantation are all issues that need to be explored.

Table 3 gives a brief summary of some retinal organoid transplantation cases in recent years. There are two transplantation methods: one is to digest the retinal

Graft/Host	Transplantation method	Transplantation result	Reference
Rhodopsin-GFP-mESC-ROs-rod precursors/adult mice with retinal degeneration	Cell suspension	Transplanted cells integrate within the degenerated retinas of mice and mature into outer segment-bearing photoreceptors	[119]
CRX-GFP-mESC-ROs-photoreceptors/adult NOD/SCID recipient mice	Cell suspension	After transplantation, the integrated cells showed typical mature rod structures with outer segments and banded synapses	[120]
mESC or miPSC-derived 3D retinal tissue/advanced retinal degeneration model (rd1) that lacked ONL	Retinal tissue	A structured outer nuclear layer (ONL) with complete inner and outer segments was developed; host-graft synaptic connections were observed	[121]
hESC-retina sheet/Two focal selective photoreceptor degeneration monkey models	Retinal tissue	Transplanted retinal tissue differentiated into a series of retinal cell types, including rod and cone photoreceptors that formed structured outer nuclear layers; formation of host-graft synaptic connections	[122]
Rhodopsin-GFP-mESC-ROs-(CD73-MACS)-photoreceptors/mouse models with mild or severe cone-rod degeneration	Cell suspension	Some cells integrated into mouse retinas and acquired a mature morphology, expressing rod and synaptic markers in close proximity to secondary neurons	[123]
Mesc-ROs-cone/adult Aipl1−/− mice	Cell suspension	Transplanted cells showed capacity to survive and mature in the subretinal space	[124]
hPSC-ROs-L/M-opsin+ cones/Nrl−/−mice or advanced retinal degeneration mice	Cell suspension	Human cones can become incorporated within an adult mammalian retina	[44]
hiPSC-ROs(cGMP)-photoreceptors/immunodeficient mouse	Cell suspension	Retinal cells successfully integrated into the photoreceptor layer and developed into mature photoreceptors	[125]
hESC-ROs-retinal sheets /mice of end-stage retinal degeneration with immunodeficiency	Retinal tissue	Long-term survival and well-structured graft photoreceptor layer maturation without rejection or tumor formation; formation of host-graft synaptic connections	[126]
hESC-ROs(30–65 days of differentiation)-retinal sheets/immunodeficient rho S334ter-3 rats	Retinal tissue	The transplanted sheets differentiated, integrated, and produced functional photoreceptors and other retinal cells; maturation of the transplanted retinal cells created visual improvements; the donor cells were synaptically active	[127]

Graft/Host	Transplantation method	Transplantation result	Reference
CRX-hiPSC-ROs-photoreceptors precursors (CD73-MACS)/P23H rats	Cell suspension	CD73+ photoreceptor precursors can be isolated in large numbers and transplanted into rat eyes, showing capacity to survive and mature in close proximity to host inner retina (hiPSC-derived retinal cells did not appear to migrate to host ONL)	[128]
CRX-hiPSC-ROs-photoreceptors precursors/Pde6brd1 mice	Cell suspension	The CRX+ cells settled next to the inner nuclear layer and made connections with the inner neurons of the host retina, and approximately one-third of them expressed the pan cone marker, Arrestin 3, indicating further maturation upon integration into the host retina	[129]
hESC-ROs-retinal progenitor cells (RPCs)/RD models of rats and mice	Cell suspension	Transplanted cells significantly improve vision and preserve the retinal structure	[130]
hiPSC-ROs-Jaws-expressing PRs/blind mice lacking the photoreceptor layer	Cell suspension	Light-driven responses at the photoreceptor and ganglion cell levels were observed	[131]
hiPSCs-ROs-Müller glia/rats depleted of retinal ganglion cells by NMDA	Cell suspension	Transplanted cells can partially restore visual function	[132]
hESC-ROs-retinal tissue/cat	Retinal tissue	Large number of graft-derived fibers connecting the graft and the host; presence of human-specific synaptophysin puncta in the cat retina	[133]
hESC-Ros-retinal sheet/immunodeficient RCS rats	Retinal tissue	The transplanted organoids survived more than 7 months; developed photoreceptors with inner and outer segments, and other retinal cells; and were well-integrated within the host	[134]
hiPSC-ROs-cones/mice with retinal degeneration	Cell suspension	Restoration of surprisingly complex light-evoked retinal ganglion cell responses and improved light-evoked behaviors in treated animals	[135]
hiPSC-ROs-retinal sheets with PLGA scaffolds/rhesus monkey	Retinal tissue	With sufficient graft-host contact provided by the scaffold, the transplanted tissues survived for up to 1 year without tumorigenesis; Histological examinations indicated survival, further maturation, and migration	[136]
mESC-ROs-retinal progenitor cells (RPC)/mice with retinal degeneration	Cell suspension	RPC grafts form active synaptic networks within sites of ADR that functionally integrate with the retinal neuron populations and that resemble physiological patterns of neural circuits to the normal retina	[137]

Graft/Host	Transplantation method	Transplantation result	Reference
ROs and polarized RPE sheets were made into a co-graft using bio-adhesives/ immunodeficient RCS rats	Total retina patch	Co-grafts grew, generated new photoreceptors and developed neuronal processes that were integrated into the host retina	[138]

Table 3.
Research on transplantation of retinal organoids.

organ into a single cell, from which the photoreceptor cells are purified and enriched, and the transplantation is carried out in the form of cell suspension. Another method is to strip the photoreceptor layer from the retinal organ and transplant it in thin slices. This method is more difficult to operate, because it is difficult to maintain the correct shape and polarity of the retina when it is transplanted into the eye of the host, and appropriate transplantation instruments need to be designed. The implant may contain some interneurons that block the connection between photoreceptor cells and the remaining inner layer of the retina in the host, and there are eye size requirements in the host animal. The implant is usually performed in rats, cats and non-human primates. In general, we have gained a lot from the exploration of retinal organ transplantation. A number of studies have shown that transplanted cells or tissues can survive in the host eye for a long time, migrate and integrate into the correct location. Integrated cells can further differentiate and mature in vivo, presenting typical cell structures, such as internal and external segments, and expressing corresponding cell markers and synaptic markers. In some studies, the formation of synaptic connections between host and graft and improvement of host visual function were also observed. In the host, transplanted cells or tissues are electrically excitatory [136], indicating their potential for restoring visual function. Through behavioral and electrophysiological experiments, we found that the host can not only slow down the progressive visual loss to some extent, but also show a relatively significant recovery of visual function [127, 130, 131, 134, 135, 137, 138]. These are exciting results and suggest that cell replacement therapy based on retinal organoids is a promising treatment that will bring light to patients with retinal diseases.

4.3 Retinal organoids for drug screening

Drug development focuses on screening, a process that requires cell models. The closer the cell model was to the physiological state, the more accurate the screening was. Therefore, organoids are undoubtedly a better choice for drug screening. Some ocular supplements, vitamin E, lutein, astaxanthin, and anthocyanin, have been reported to protect retinal photoreceptor degeneration induced by 4-hydroxytamoxifen (4-OHT) and light [139]. Few studies have successfully screened effective drugs using retinal organoids. In addition, there are some studies using retinal organoids as screening tools to explore the membrane transport effects of some microbial opsin [140]. These results suggest that retinal organoids can be used to validate the effectiveness of some therapeutic products and drug molecules.

5. Limitations and deficiencies

In recent years, although the technology of retinal organoids has made great progress, it is still beyond the reach of our existing tools and technologies to construct retinal organoids with the same structure and physiological functions as the mature retina in vivo. Our research on retinal organoids is still in its infancy and there are some limitations to overcome.

Long-term maintenance of retinal organoids depends on oxygen and nutrients, and in our existing culture system, oxygen diffusion limits the size of retinal organoids and the development of their internal cells, especially ganglion cells. Currently, we are trying to introduce a combination of tissue engineering techniques to solve this problem, such as the use of bioreactors and retinal chips [62, 63, 65]. The absence of vascular system also limits the long-term maintenance of organoids. Microglia are the resident immune cells of the central nervous system and are particularly important for the development of the retina, which can regulate the survival of neurons and prune synapses [141]. Co-differentiation of retinal organoids and vascular tissues or microglia in a dish is challenging because they come from different germ layers. The retina develops from the ectoderm, while vascular tissue-associated cells and microglia originate from the mesoderm. Therefore, we usually choose to achieve the complexity of retinal organoids through co-culture. In recent years, the realization of vascular structure in human brain has made some progress. After transplantation of human cerebral organs into the cerebral cortex of mice, the growth of blood vessels in mice was induced to increase the survival and maturation of cells [142]. In vitro, a study found that ectopic expression of human ETS variant 2 (ETV2) in hESCs can form a complex vascular-like network in human cortical organs and promote the maturation of organoid function [143]. In the future, we also expect that 3D printing of vascular tissue [144] and co-culture with mesodermal progenitor cells [145] will make the differentiation system of retinal organoids more perfect. For the retina in a petri dish to function, the most important point is to establish synaptic connections and form functional circuits. While our differentiated retina can form synaptic connections now, it's not nearly as good as the complex network of synapses in the retina in vivo. Even retinal organoids derived from normal stem cell lines respond poorly to light. This may be due to the gradual degeneration of ganglion cells during late differentiation and lack of connection to the brain, which hindrance our assessment of retinal functional circuits. It may also be associated with limited growth of the outer segment due to the lack of direct interaction with RPE. Retinal organoid technology has solved the problem of cell-cell interaction, but in organisms, tissue-to-tissue and organ-to-organ interactions remain important for development. For example, the relationship of the retina to the lens, ciliary body and cornea, and the relationship of the retina to the brain.

6. Conclusions

It's an exciting time, and technological advances have made a lot of things possible. Retinal organoids are our research tools for overcoming retinal diseases. It allows us to further understand the development and maturation of the retina, reproduce disease pathology and phenotypes in vitro, and explore the feasibility of gene therapy. In addition, it provides us with cells for cell transplantation and drug screening. We have enjoyed the great benefits brought by retinal organoids. However, their defects

and deficiencies are also prominent, which is the direction we need to work towards. There is still a long way to go in the development of retinal organoids, and we expect that technological breakthroughs will enable us to advance to the next level in this field in the future.

Acknowledgements

We thank Dr. Ze-Hua Xu for providing photos of retinal organoid differentiation.

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