



Organoids and organ chips in ophthalmology

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ABSTRACT

Recent advances have driven the development of stem cell-derived, self-organizing, three-dimensional miniature organs, termed organoids, which mimic different eye tissues including the retina, cornea, and lens. Organoids and engineered microfluidic organ-on-chips (organ chips) are transformative technologies that show promise in simulating the architectural and functional complexity of native organs. Accordingly, they enable exploration of facets of human disease and development not accurately recapitulated by animal models. Together, these technologies will increase our understanding of the basic physiology of different eye structures, enable us to interrogate unknown aspects of ophthalmic disease pathogenesis, and serve as clinically-relevant surrogates for the evaluation of ocular therapeutics. Both the burden and prevalence of monogenic and multifactorial ophthalmic diseases, which can cause visual impairment or blindness, in the human population warrants a paradigm shift towards organoids and organ chips that can provide sensitive, quantitative, and scalable phenotypic assays. In this article, we review the current situation of organoids and organ chips in ophthalmology and discuss how they can be leveraged for translational applications.

Introduction

Loss of vision severely affects the quality of life and is one of the most feared health conditions in society. The demand for basic research on ophthalmic diseases is rising due to the increasing world population and the increase in life expectancy and subsequent rise in the number of

cases of ophthalmic diseases such as corneal dystrophy (e.g., Fuchs'), dry eye disease, Sjögren's syndrome, retinitis pigmentosa (RP) and retinoblastoma. Previously, experimental studies have been performed *in vivo* on animal models (from *Drosophila* and zebrafish to rodents and large mammals) or *in vitro* cell culture models [1–3]. These models have contributed to ophthalmology in many ways, including their role in

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finding basic functional characteristics at the cellular level, modeling eye development, disease pathogenesis, therapy development, and drug screening [4–9]. However, these studies can suffer from limited clinical translatability, for example, due to species differences in the tropism of viral vectors or the transcriptome for both protein-coding and non-coding transcripts [10–12]. The commonly used *in vitro* models often fail to reproduce organ-level functionality, which is needed for realistic disease modeling. Therefore, more representative models of human eye tissues are required [13].

In response to this need, two new technologies have been recently developed: organoids and organ-on-chips (organ chips) [13,14]. Organoids are stem cell-derived, three-dimensional assemblies that consist of organ-specific cell types that self-organize through cell sorting and spatially restricted lineage commitment, thus mimicking the architectural and functional complexity of native organs [15]. Organoids are derived from embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), or organ-restricted adult stem cells (aSCs). During stem cell differentiation, temporal manipulation of the *in vitro* microenvironment through supplementation with exogenous components such as growth factors, small molecules, and extracellular matrix (ECM) substrates can simulate conditions of the fetal microenvironment, and improve organoid differentiation and maturation [15]. Another evolving model is organ chip technology, which combines the uses of microfluidic technology, biomaterials, and cell culture techniques to model human-like organs on a micro-scale. Organ chips are microchips designed to recapitulate the microarchitecture and function of living human organs. Commonly, organ chips are transparent, contain varying hollow microfluidic channels and cell compartments which can be lined by living human organ-specific cells. External artificial forces can be applied to mimic the physical microenvironment of the living organs. Organoids and organ chips have been developed to model many organs, including the gut, liver, kidney, brain, and more recently, eye structures including the retina, cornea, and lens [16]. In this article, we discuss the use of organoids and organ chips in ophthalmology. We review the use of these techniques to study eye development and physiology, disease mechanisms, and the development of diagnostic assays and therapeutics for personalized medicine.

Cornea

The cornea is the transparent outermost layer of the eye that is responsible for focusing most of the incoming light and is thus essential for vision. Over the last decades, efforts have been made to create *in vitro* corneal models. In 1993 Minami et al. succeeded in reconstructing the three layers of the cornea (endothelium, stroma, and epithelium) from cells isolated from bovine cornea tissues [17]. The three layers were engineered sequentially on top of each other, with the epithelial layer interfacing a liquid/air matrix. A similar approach was employed to develop the 3-dimensional corneal model derived from human cells by Germain et al. that reconstructed corneas by culturing epithelial cells on collagen gels containing fibroblasts [18]. Griffith et al. fabricated a human corneal model employing human corneal cell lines. They found that their model resembled the human cornea in morphology, transparency, and histology. The model responded to both stromal swelling and corneal wound healing to a degree similar to post-mortem cornea [19]. The efforts to develop the 3D cornea model entered a new phase with the introduction of organoid technology and chip-based modeling. In what follows, we discuss corneal organoids and cornea chips, and then describe their applications to drug studies.

Corneal organoids

Corneal organoids are 3D cornea models designed to be suitable for developmental studies, modeling of some cornea disorders, and possibly organ replacement purposes [20]. Organoids are generated from embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) that

differentiate into various cornea cells [21–24].

In a breakthrough study, Foster et al. used human iPSCs to develop corneal organoids. These organoids contained three cell types of the cornea - epithelium, stromal keratocytes, and the endothelium. These structural features were characterized using immunofluorescence staining for epithelial markers (KRT3, KRT14, and p63), stromal keratocytes (CD34), and endothelial markers (COL8A1, F11R, S100A4). In addition, these organoids consisted of extracellular matrix collagens and proteoglycan core proteins which are essential components of the stromal matrix. TEM and immunofluorescence staining showed different matrix proteins and organized protein fibrils in different layers of the organoids. While the outer layers contained perlecan and collagen type VIII, the deeper layers stained for stromal proteins LUM and KERA, Collagen types I and V [25].

Shortly after the development of corneal organoids by Foster et al. a new protocol was developed by Susaimanickam et al. which generated, using a simpler and more efficient culture method, more complex 3D corneal organoids and even, in rare cases, whole eyeball-like structures. In this protocol, either human ESCs or human iPSCs are cultured *in situ* in retinal differentiation medium (RDM), with the absence of noggin. After four weeks in culture, the stem cells had differentiated in eye field primordial clusters (EFPs). They found that continued *in situ* differentiation of these EFPs in RDM led to the formation of lens epithelial clusters, ocular surface epithelium, and optic cups. Interestingly, they found that in rare cases the EFPs gave rise to 3D miniature eyeballs, including an anterior transparent cornea primordium surrounded by the neuroretinal cup. At two weeks of suspension culture, the EFPs gave rise to both retinal and corneal primordia. At this point, the corneal primordia regions were lifted and cultured in the corneal differentiation medium (CDM) for further maturation. Six to eight weeks after culturing the corneal primordia gave rise to minicorneas. Subsequent examination of these minicorneas showed prevalent corneal morphological structures and expressed cornea-specific markers [26].

These corneal organoids open up possibilities for multiple downstream applications that are unattainable using current *in vitro* culture systems.

Cornea chips

Engineering cornea mimics on chips is still at its infancy. A few steps have been taken towards modeling cornea; despite their simplicity, current cornea chips are already able to recapitulate certain disease features. Puleo et al. made the first attempt to culture cornea cells in microfluidic devices in 2009 [27]. They succeeded in developing a bilayer structure containing a corneal epithelial layer and a layer of stromal cells on a collagen vitrigel substrate. The model was then used to measure transepithelial permeability. Nearly a decade later, Bennet et al. developed a cornea chip that included epithelial layers, basement membrane, and Bowman's membrane, and importantly, they simulated tear flow dynamics in their microfluidic device [28]. They used immortalized human corneal epithelial cells (hTCEpi) and cultured them on a Polydimethylsiloxane (PDMS) membrane. They found that cells seeded on a fibronectin-coated membrane were more viable compared to uncoated or collagen-coated membranes. The permeability of the epithelium underflow closely resembled *in vivo* conditions.

A significant breakthrough in the development of an ocular surface chip was the chip development by Seo et al. that integrated both cornea and conjunctiva structures in one platform and interfaced them with a blinking eyelid [14]. They developed a dome-shaped scaffold with a perfusion system and an eyelid mimic that is actuated to slide on the scaffold. The cornea mimic included epithelial cells with primary human keratocytes embedded in a hydrogel to mimic the stroma. Using a 3D patterning approach, concentric patterns of the conjunctiva (including epithelial and goblet cells) and cornea structures were fabricated. The structure, however, lacked vasculature and immune cells that are present in the conjunctiva *in vivo*.

The ocular surface chip developed by Seo et al. recapitulated important anatomical and physiological properties of human counterparts [14]. Stratified epithelium in this device is composed of 7–8 layers of epithelial cells (similar to *in vivo* conditions) as well as a layer of cells expressing basal cell-specific marker (p63). Similarly, the engineered conjunctiva contained multiple epithelial layers and showed the expression of key biochemical markers. Importantly, the tissue could produce mucin proteins. An essential aspect of this design is that the model incorporates key mechanical processes including blinking with physiological frequencies (e.g., 0.2 Hz) as well as tear film dynamics. The tear secretion rate of the device was properly tuned to its natural values, and a drainage system for tear excess was designed. This ensured the maintenance of a very thin and uniform tear film of approximately 6 μm thickness (within the range of *in vivo* values), as shown by optical coherence tomography. The blinking process was shown to be noninvasive to the ocular surface and did not lead to scarring. The mechanical shear was shown to promote cellular differentiation in the epithelial layer, indicating that the eyelid is more than just a “lid” and the mechanical forces are sensed and responded to by the cellular biochemical network.

Ocular surface chips remained to be adapted and used as disease models. The only major development in this direction was the engineering of a Dry Eye Disease (DED) model on the platform developed by Seo et al. [14]. By reducing the frequencies of blinking from 12 to 6 times per minute and adjusting the humidity of the environment allows for the simulation of an evaporative DED model. Reduced blinking frequency leads to changes in tear osmolality and film instability, as shown by break-up time estimation. Importantly, these DED related biomechanical changes led to cellular changes seen in DED *in vivo*. For example, inflammatory cytokines such as interleukin 1 β , TNF α , and matrix metalloproteinase (MMP)-9 were overexpressed upon the

reduction of blinking frequency in the chip. Overall, this proof-of-concept study demonstrates that the chip model can capture the mechanical and biochemical features of DED (Fig. 1). These results are highly promising and set the stage for the development of chip-based models for other ocular surface diseases. For this aim, one may have to incorporate additional complexities e.g. the immune system and vasculature.

Applications to drug studies

Cornea chips have been used to study pharmacokinetics and pre-clinical drug evaluation Bennet et al. performed a drug study on their model with Pred Forte (Prednisolone 1%) and Zaditor (Ketotifen) to assess the functionality of their model in drug permeability [28]. They found that the pulsatile tear flow had the most similarity to the human eye than the continuous flow or static condition [28]. Their findings were correlated with the formulation of the drug and mode of the action of the chip (static or with the flow) so that it would be a representative model of the human cornea *in vitro* [28]. Unfortunately, pharmacological immunomodulation of the ocular surface cannot be accurately mimicked using current chip designs, as these designs lack the immune system. However, nonimmunological processes can be drugged effectively in chip models of ocular surface inflammatory diseases. An example of such a study is the analysis conducted on the DED model of Seo et al. [14]. They exposed the DED model to endogenous lubricin to assess whether the findings of this *in vitro* model are comparable with clinical outcomes. The results not only showed increased break-up time and decreased area of the film tear rupture but also changed corneal fluorescein staining resembling what was found in the clinical trials. This analysis shows the values of cornea chips, as these “clinical” measurements are now possible at the preclinical stages of drug analysis,

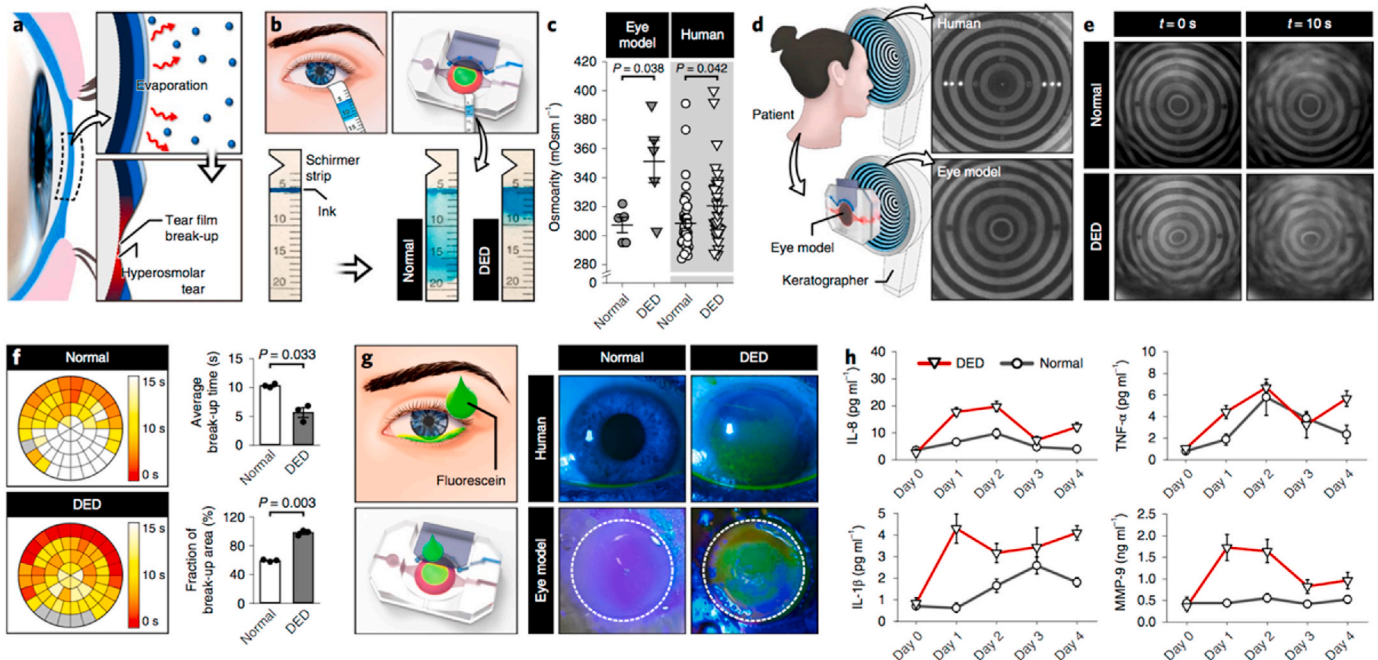


Fig. 1. Dry eye disease chip. a) Evaporation induced break-up of the tear film and increase of tear osmolality together lead to a loss of homeostasis. b) Absorption of tears into the Schirmer strips in the healthy and DED models. Tear absorption is visualized by the smearing of the blue ink within the strips. c) Tear osmolality in the DED (closed triangle) and the healthy (closed circle) models. Human clinical data of osmolality are from healthy (open circle) and DED subjects (open triangle). d) Keratographs showing concentric rings projected on the human ocular surface (top) and the engineered ocular surface (bottom). e) Representative images of projected ring patterns on the engineered ocular surface of the healthy (top row) and the DED (bottom row) groups captured at $t = 0\text{ s}$ (left column) and $t = 10\text{ s}$ (right column). f) Spatial mapping of tear film break-up time in the normal (top) and the DED (bottom) models. Different colors in the representative circular heat maps indicate different tear break-up times. g) Fluorescein staining of the eye model and human subjects. h) Concentrations of inflammatory mediators (IL-8, TNF- α , IL-1 β , and MMP-9) in the normal (circle) and the DED (triangle) groups plotted against the duration of culture. The figure is taken from Jeongyun Seo et al. with permission [14]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

thanks to the chip technology. Furthermore, the DED model was used to discover the therapeutic effect of lubricin at the molecular scale. Concentration profiles of inflammatory factors such as toll-like receptor-4 (TLR-4) of IL-8, TNF- α , IL-1 β , and MMP-9 can be measured in the tear film. The DED model showed a marked decrease in inflammatory markers upon lubricin administration, in agreement with the findings of clinical trials.

One important issue in drug assays on cornea chips relates to drug permeation and accessibility to ocular surface layers. Bai et al. created a cornea chip model to determine dextran diffusion permeability across the corneal barriers [29]. They used dextran with several distinct molecular weights ranging from 10 kDa to 70 kDa to simulate drug diffusion across cornea layers and to measure permeability. They found that epithelium is a major determinant of drug transportation rates. Future studies are needed to investigate the translational value of these drug transport data. This is because the proof-of-concept analysis by Bai et al. was based on mouse cornea cells and did not include the cellular complexity of human cornea and its collagen content precisely.

Lacrimal glands

The lacrimal glands (LGs) secrete tear fluids, which contain water, electrolytes, and various secreted substances to the ocular surface. Tears play physiologically important roles in maintaining the homeostatic environment on the ocular surface epithelium, such as lid lubrication, hydration, antimicrobial activity, and protection of the ocular surface epithelium. Tear shortage from LGs, which is induced by aging and various pathological conditions, causes dry eye disease (DED). DED is defined at TFOS/DEWS II as a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film, and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles. Recently, a therapeutic concept of functional lacrimal gland restoration using regenerative medicine, including organoids from pluripotent stem cells, has emerged as a possible way to

treat severe DED.

Lacrimal gland organoids

One of the challenges for the lacrimal gland regeneration is to reconstruct secretory gland structures and coordinated organ systems with peripheral tissues, such as nerves and blood vessels, because organogenesis requires a complex process involving tissue self-organization, cell-cell interactions, signaling with various molecules for correct cell differentiation to form organs [30,31]. A method in biotechnology based on knowledge from developmental biology for 3D stem cell manipulation *in vitro*, which is designated to mimic organ germ formation during organogenesis, allowed us to generate bioengineered organ germs, including teeth, hair follicle, and salivary glands and LGs, by using embryo-derived organ inductive stem cells (Fig. 2a) [32–37]. The bioengineered lacrimal gland germs, which were generated with compartmented cells of epithelial and mesenchymal stem cells isolated from mouse lacrimal gland germs in a collagen gel, initiate developmental processes involving the self-organization and multiple cellular assembling to form tubule-alveolar structures *in vitro* (Fig. 2b). The bioengineered lacrimal gland germs achieve mature lacrimal gland structures and recapitulate their connection with peripheral tissues and innervation for fully tear-secretion functions after orthotopic engraftment *in vivo* (Fig. 2c and d). As reported in the bioengineered teeth regeneration study, the development of a method to mature the bioengineered lacrimal gland germs *in vitro*, which can immediately function the bioengineered organs *in vivo*, will contribute to lead future organ replacement therapy for DED [34]. These studies have demonstrated that the functional bioengineered lacrimal glands for organ replacement therapy can be achieved by reproduction of the developmental process of LGs in organogenesis.

An organoid as a partially functional mini-organ can be generated by using specific developmental gene expression and cytokine signaling, which induce self-organized body patterning and subsequent organ-forming field from pluripotent stem cells [38–43]. Recently, fully

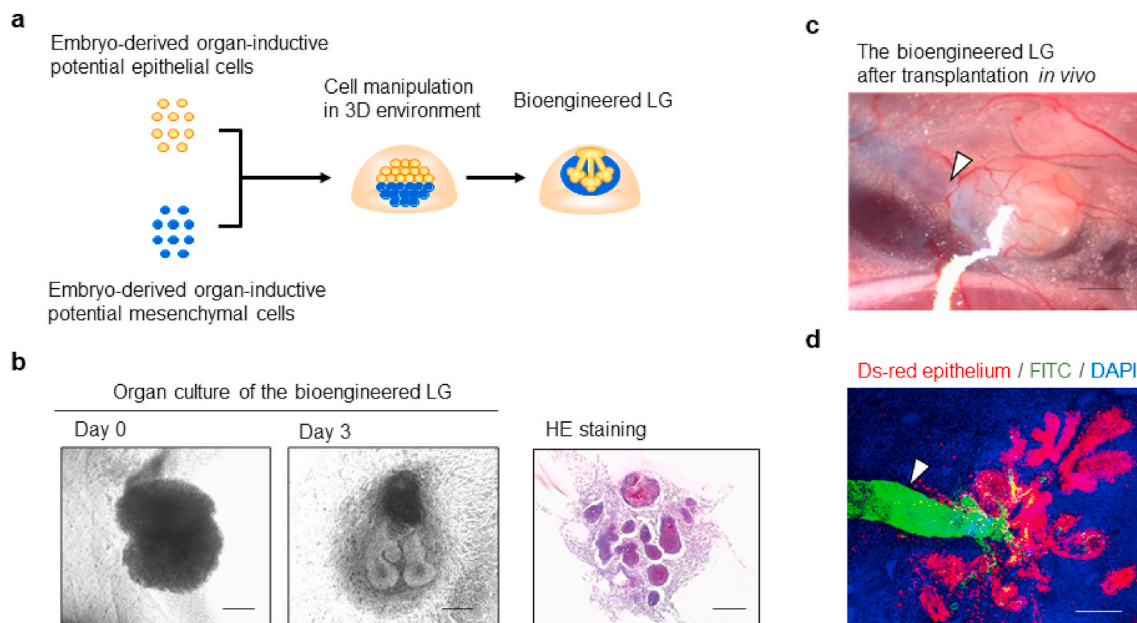


Fig. 2. Development of the bioengineered lacrimal glands *in vitro* and *in vivo*. (a) Functional lacrimal gland regeneration using mouse embryo-derived organ-inductive stem cells in the 3D culture environment. (b) Organogenesis of the bioengineered lacrimal glands with branching morphogenesis *in vitro* (left and center panels) and an image of HE staining of the bioengineered lacrimal gland (right panel). scale bar, 200 μ m. (c) Functional lacrimal gland replacement after orthotopic transplantation *in vivo*. Arrowhead shows the connection between the host lacrimal excretory duct and the bioengineered lacrimal gland. Scale bar, 500 μ m. (d) The bioengineered lacrimal glands developed with full functionality. Ds-red expressing epithelium achieved acini and duct structures. FITC gelatin (green), which was injected from the host lacrimal excretory duct, reached to the bioengineered lacrimal gland. Arrowhead shows the connection between the host lacrimal excretory duct and the bioengineered lacrimal gland. Scale bar, 200 μ m. The figures were reprinted from Refs. [48]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

functional salivary glands from mouse ES cells have been generated through the induction of organ-forming field (the oral ectoderm; for the salivary glands) with the expression of specific genes for salivary gland development [44]. In the field of the lacrimal gland regeneration, a set of transcription factors, which plays a role in the lacrimal gland development, has been clarified to induce the organ-forming field and signaling for the lacrimal gland organoids [45,46]. As well as the possibility of tissue stem cell injection therapy reported previously [47], the further development of this method of organoid technology for LGs will contribute to the realization of lacrimal gland regenerative medicine.

Lacrimal gland chips

Various innovations in bioengineering have been applied to regenerate functional organs to treat or modeling diseases. For example, artificial regulation of tear secretion from LGs by stimulating neural pathways using medical devices has been proposed [49]. To generate complex secretory gland structure, previous reports have used biohybrid materials, such as a decellularized scaffold, for a successful 3D reconstruction of LG [50,51]. Other studies have reported the *in vitro* models of several components of the ocular surface, such as conjunctival tissues and lacrimal gland epithelium [52], individually [53]. The 3D culture environment, including microgravity cell culture and Matrigel culture methods, has been a key to investigate the lacrimal gland function and DED [54–56]. A tissue engineering technology for reconstruction of a complex 3D organ system (LGs-tear film-ocular surface) has been attempted to build up *in vitro* disease modeling of DED [57]. These studies have indicated that it is effective to create functional tissue mimics, which use a co-culture system of various tissues of tear film-ocular surface system including the lacrimal gland spheroids and conjunctival epithelium, as a model for DED and therapeutic evaluation. The next-generation research of the lacrimal gland regenerative medicine using organoids and organ chips is now expanding as a viable model based on recent exponential advances in developmental biology, stem cell biology, and tissue engineering technology.

Retina

Retinal organoids

The first retinal organoids (ROs) were made from mouse (m)ESCs [13,58]. The Sasai laboratory found that the development of the optic cup is a self-directed process and does not rely on influence from external structures. Their model had the six major types of neurons and one glial cell type of the retina and could recapitulate many aspects of retinal function, including morphogenetic movements, interkinetic nuclear migration, and apical-basal polarity [13,58]. Although their model was not perfect in recapitulating all facets of the adult mouse retina, including a low percentage of cone photoreceptors, it was a milestone in generating more advanced retinal models that recapitulated *in vivo* characteristics of the mouse retina. Further optimization of their protocol enabled the generation of human (h)ESC-derived ROs for the first time (Nakano et al.) [40]. Compared to the stem cell-derived RO mouse model, the retina derived by this method had longer culture time, which is due to the inherent differences in gestational periods between species. The methodology used was based entirely on a 3D approach relying on extrinsic modulation of cell signaling pathways. Starting from a single cell suspension of hESCs, the cells are seeded in equal numbers in V-bottomed wells of a 96-well plate and undergo quick aggregation to form embryoid bodies (EB). EBs undergo neural induction and form optic vesicles, which are subsequently kept in suspension culture for maturation to laminated ROs [40]. The main alternative to this methodology, popularized by Zhong et al., requires far less extrinsic modulation of cell signaling pathways and proceeds in a mostly self-directed process. Here, hiPSCs are grown to near confluence and made into small floating aggregates, either chemically or mechanically, in suspension to

form EBs which undergo neural induction. Subsequent plating of EBs allows for the formation of the retinal neuroepithelium, which can be individually dissected from the bottom of the well, or alternatively, the whole contents of the well can be lifted after mechanically scoring the well in a grid pattern. This is followed by the sorting of ROs for long term suspension culture where they undergo lamination and maturation [59, 60]. An alternative to this approach is to let hiPSCs grow to confluence and, instead of generating small floating aggregates, remain as adherent cultures. Removal of FGF2 from the culture medium initiates spontaneous differentiation which is followed by the promotion of neural induction and retinal neuroepithelium formation [61,62]. The methodology by Zhong et al. was the first to show fully laminated 3D iPSC-derived retinal tissue which also yielded more developed outer-segment structures that responded to light stimuli [59]. A third methodology by Lowe et al. used small aggregates of hESCs embedded in Matrigel to form single-lumen epithelial cysts that subsequently adhere to the culture surface forming colonies of retinal progenitors. Dispase treatment promoted lifting of these colonies, which in floating suspension culture formed laminated and maturing retinal organoids [63]. The use of hESCs and hiPSCs has been successfully implemented using all three methodologies, and continued modifications and optimizations are being implemented [40,59,60,64–68]. The retinal architecture of the human fetal retina is recapitulated in hiPSC retinal organoids (Fig. 3).

Organoids are useful for understanding the developmental physiology of the retina and its underlying transcriptomic mechanisms. Here, we provide some examples of RO implementation for these purposes. Previously, an essential role of thyroid hormone signaling on cone cell viability and opsin expression has been established. However, the exact mechanism of cone cell differentiation was not known [69]. Retinal organoids have been implemented to further elucidate this mechanism. Eldred et al. found that the retina plays an active role in specifying S or L/M cone subtypes through the temporal expression of thyroid hormone degrading and activating proteins. In early retinal development, thyroid hormone levels are kept low which initiates differentiation to S cones, later in development thyroid hormone levels rise to specify L and M cone cells [70].

The organoid models can be used for studying the effect of different genes in eye formation and neuroretina differentiation. Takata et al. studied the effect of *R-spondin 2* (*Rspo2*) and *Sine oculis*-related homeobox 3 (*Six3*) genes [71]. They used *Six3*^{-/-} miPSC-derived and *Six3* conditional knockout (CKO) mESC-derived organoids, finding that *Six3*-mediated suppression of *Rspo2* is necessary for neuroretina differentiation and optic vesicle morphogenesis [71].

Capowski et al. studied the expression pattern of microphthalmia-associated transcription factor (MITF) during differentiation in their hESC-derived optic vesicle model. MITF is a vital regulator of pigmented cell survival and differentiation. They found that in addition to the role of MITF in retinal pigment epithelium (RPE) development, it is also crucial for early optic vesicle cell proliferation [73]. Quinn et al. used hiPSC-derived ROs and human fetal retina to investigate the onset of expression of CRB1 (Crumbs homolog-1) and CRB2 during development and early maturation of the retina [72]. Mutations in CRB1 are associated with a spectrum of retinal dystrophies including RP type 12 and LCA type 8 [74–78]. They found that during the first trimester, CRB2 is the predominant CRB family member. In contrast, the onset of expression of the canonical CRB1 protein at the subapical region coincides with the maturation of the retina during the second trimester. This pattern of CRB1 and CRB2 expression was recapitulated in ROs [72]. To verify that retinal ganglion cells (RGCs) can be identified by the expression of specific surface antigens. Aparicio et al. compared surface antigen expression patterns in the human fetal retina and hESC-derived ROs. They found that CD184 and CD171 were expressed in RGCs from both tissues and that early post-mitotic RGCs express high levels of CD184 while CD171 expression is found in maturing RGCs. These cell surface markers could then be used to purify the RGC population using flow cytometry [79]. RGCs derived from ROs can be used as an effective

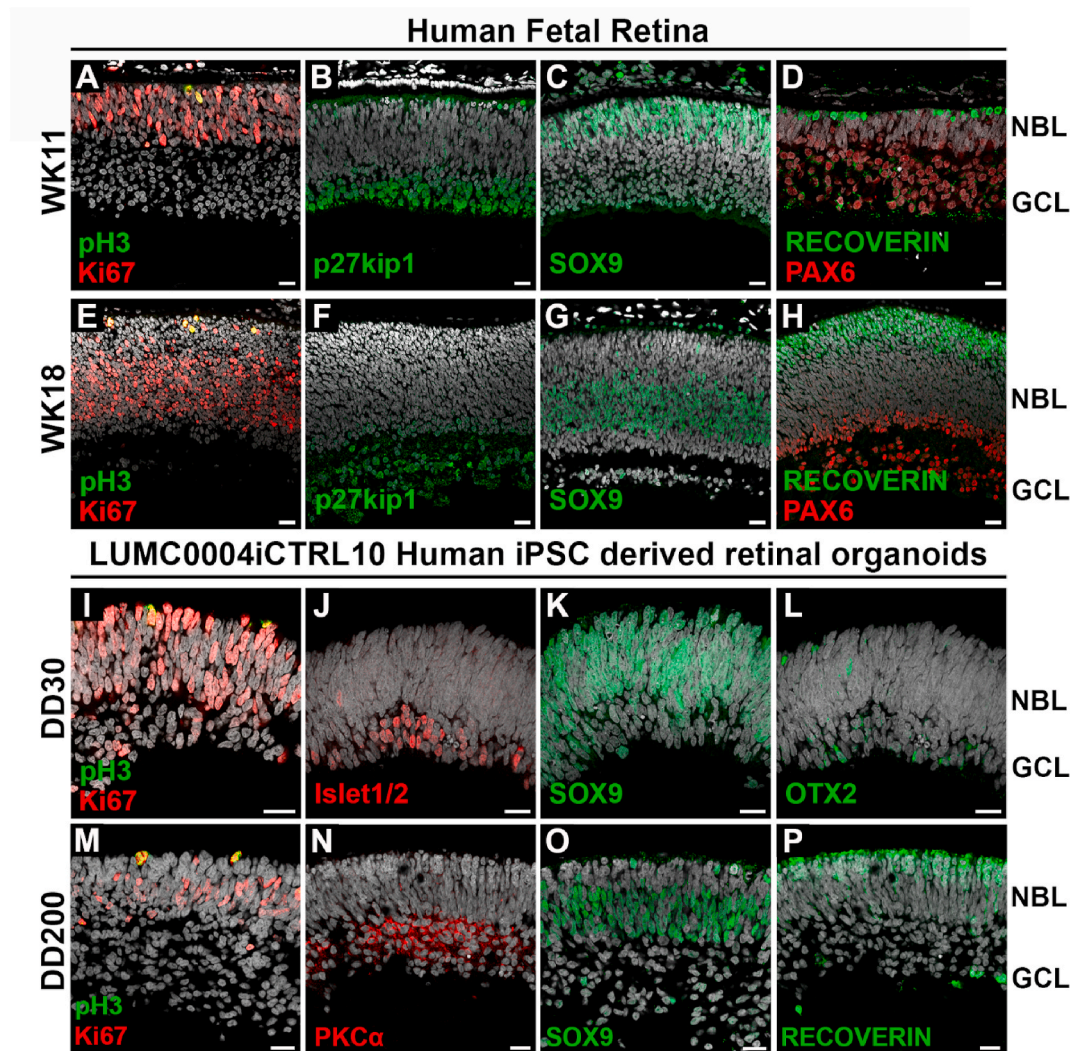


Fig. 3. Retinal architecture in the human fetal retina and human iPSC retinal organoids. Immunohistochemistry pictures of WK11 (A–D) and WK18 (E–H) human fetal retina and DD30 (I–L) and D200 (M–P) LUMC0004iCTRL10 hiPSC-derived retinal organoids. Sections were stained with antibodies against: Ki67 (A, E, I, M), pH3 (A, E, I, M), p27kip1 (B, F), SOX9 (C, G, K, O), Recoverin (D, H, P), PAX6 (D, H), Islet 1/2 (J), PKC α (N), OTX2 (L). In human fetal retina at week 11 in the 1st trimester of pregnancy, we observed cycling cells that stained positive for anti-Ki67 and spanned the thickness of the neuroblast layer (NBL). The mitotic cells located most apically and stained positive for pH3 (A). Inner retinal cells as marked by p27kip1 and PAX6 were restricted to the ganglion cell layer and a subset of cells in the NBL (B and D). The cells that exited the cell cycle were marked with p27kip1, whereas the ganglion cells, amacrine cells, and migrating retinal progenitors were marked with PAX6. Radial glial progenitor cell nuclei spanned the thickness of the NBL and stained positive for anti-SOX9 (C). Newborn cone photoreceptors marked positive for anti-recoverin (D). In the human fetal retina at weeks 18 in the 2nd trimester of pregnancy, we observed the localisation of anti-pH3-positive mitotic cells most apically within the NBL (E). However, the cycling anti-Ki67-positive cells became mostly restricted to the middle NBL cells but also labeled occasional outer NBL cells (E). Both p27kip1- and PAX6-positive cells restricted to the inner NBL and the ganglion cell layer (F, H). SOX9-positive cell nuclei localised in the middle NBL and occasionally the outer NBL, marking maturing Müller glial cells (G). The outer NBL showed an increase in recoverin-positive photoreceptors at week 18 compared to week 11 (H). In early DD30 hiPSC retinal organoids we observed that Ki67-positive cycling cells spanned the thickness of the NBL with pH3-positive mitotic cells located most apically (I). Ki67-positive cycling cells were also detected in the ganglion cell layer (GCL). Islet1/2-positive cells were found mostly restricted to the GCL with sporadic cells in the NBL (J). SOX9-positive radial glial progenitor cell nuclei spanned the thickness of the NBL but were also seen occasionally in the GCL (K). Immature photoreceptors that stained positive for anti-OTX2 could be found in both the NBL and GCL (L). In later DD200 hiPSC-derived retinal organoids Ki67-positive cycling cells restricted mostly to the middle NBL but occasionally were detected in the outer NBL and in the GCL, whereas pH3-positive mitotic cells located apically (M). In the inner retina PKC α -positive bipolar cells (N) were detected. SOX9-positive cell nuclei became more restricted to the middle NBL but occasionally were detected in the outer NBL and the GCL (O). Recoverin-positive photoreceptor cells were mostly restricted to the outer NBL. Some recoverin-positive cells were detected within the NBL and occasional recoverin-positive cells were detected in the GCL (P). Scale bars: (A–P), 20 μ m. Adapted from Quinn et al. 2019 [72] under a creative commons license.

platform to investigate RGC development, organization, and neurite outgrowth [80].

ROs have been used to profile the dynamic transcriptional landscape of retina from early progenitors to differentiated retinal cell types [81–83]. These transcriptomic data have been achieved by using different reporter lines, each assessing a specific type of cell in the human retina. Hereby nine different clusters of cells have been observed during the differentiation of ROs. It has been found that mitotic cells and

RGCs decrease over time, rod and cones are formed afterward, and Müller glia increases towards day 200 [84]. Neural retina leucine zipper (NRL) is a gene that has a critical role in rod photoreceptor formation, and patients with NRL mutations exhibit rod degeneration enhanced S-cone syndrome and RP. ROs of null NRL models have also been developed to study the cone photoreceptor development in human-derived ROs [85]. These models can also be useful to assess the transcription factors involved in photoreceptor development.

Lastly, in addition to radial glial progenitors, a peripherally located stem cell-like niche called the ciliary marginal zone (CMZ) can contribute to the production of postmitotic cells to the retina in mammals [76,86–88]. The Sasai laboratory used an induction reversal method to generate human ROs with RPE, which contained a CMZ at the boundary between these two tissues. This region contained CM-like stem cells, which were able to contribute to retinal expansion by *de novo* generation of retinal progenitors [89]. Further exploration of the mechanisms underlying CM-like stem cells will help advance ocular regenerative therapeutics.

Retinal organoids and disease modeling

In recent years, retinal organoid models of various eye diseases have been created. These models can be either used for studying the underlying pathomechanisms as well as for therapeutic purposes. Table 1 summarizes some RO models of retinal diseases that have been developed thus far. However, some questions are arising regarding the further applicability of organoid models for disease modeling. Points to consider include whether the developmental “age” of the ROs corresponds to when the patient would get disease onset typically, or whether the culturing microenvironment exacerbates disease characteristics in organoid culture are concerns that need to be addressed in the upcoming studies. Modeling of the late-stage disease will be a particular challenge due to the immaturity of ROs even after long-term cell culture, the addition of stressors to induce aging phenotypes should be explored. Furthermore, ROs are still relatively naive structures that lack optic nerve, retinal vasculature, and microglia.

Applications of retinal organoids to drug studies

For drug studies, human RO technology promises to be a more realistic model of human development and disease than animal and 2-dimensional cell culture models [104,105]. RO models can be used to evaluate targeted therapy and drug toxicity in the pre-clinical drug development stage [106]. In what follows, we discuss two examples of RO-based drug studies. First, a model of advanced retinoblastoma tumor organoids (resembling retinal tumors and seeds) created by Saengwimol et al. was used to assess cell cycle arrest in response to drugs and their genotoxic effects. The model was used to compare the outcome of single drugs and their combinations. In the first step, they evaluated whether the result of this model will be comparable to clinical outcomes. Drugs commonly used in intravitreal chemotherapy, including melphalan, topotecan, and methotrexate were used with various concentrations and 24–72(h) exposure times. Low dose melphalan (8 and 16 μM) with prolonged exposure time showed a similar effect to the high dose of melphalan (32 μM) with short exposure time, leading to induced S-phase arrest and reduced G2/M-phase. Topotecan (11 μM) efficiently reduced the number of tumor cells in G0/G1 and G2/M phases. Methotrexate showed the least anticancer effectiveness. Methotrexate reduced the G0/G1-phase cells and increased cell death in the sub-G1 phase, however, S and G2/M phases were activated. The results were comparable to clinical results indicating the translatability of RO data. As topotecan and melphalan showed similar effects, one may ask whether their combination will be a desired therapeutic strategy. Therefore, the researchers took a second step to analyze targeted therapy and the genotoxicity of a combination of topotecan (11 μM) and melphalan (16 μM), which was challenging to perform in clinics. The combination not only had a profound influence on subretinal seeds or recurrent retinal tumors but also targeted proliferative tumor cones effectively [102]. However, a combination of melphalan and topotecan was more genotoxic than melphalan alone. This study thus shows the power of the RO model in addressing therapeutic challenges.

In another study, Ito et al. used miPSC-derived ROs as a model for reproducing photoreceptor degeneration treated by 4-hydroxytamoxifen (4-OHT) and diethylstilbestrol (DES) individually that induces

acute cell death. Additionally, they established a fluorescent live-cell imaging system in order to evaluate their induced damage model. Those side effects were already confirmed in the mouse retinal explant experiment. Therefore, ROs can recapitulate the drug side effects. They also analyzed the protective effect of vitamin E (400 μM) and Lutein (200 nM) as ophthalmic supplements for treating the photoreceptor degeneration. The result showed the superior efficacy of vitamin E compared to Lutein in suppressing oxidative and endoplasmic reticulum (ER) stress-related gene [107]. Overall, ROs have been used in several proof-of-concept drug studies with promising results. Nevertheless, there are several shortcomings, such as the lack of vascularization and immune cells. Thus, current RO models cannot be used in drug studies such as screening of neuroprotective drug development for retinopathy, where incorporation of inflammatory cells is essential [105].

Applications retinal organoids to transplantation therapy

Pluripotent stem cells can be used to provide a potentially unlimited supply of retinal specific cell types. Retinal organoids derived from hESCs and hiPSCs can serve as a cellular source for transplantation purposes, either as a retinal cell suspension or an organized retinal sheet [108–111]. Most studies typically focus on the use of ROs as a source for rod and cone photoreceptors, but they have also been used as a source for retinal ganglion cells and Müller glial cells [62,109–113].

In 2016 a string of studies highlighted the need for reevaluation of previous photoreceptor donor transplantation studies due to cytoplasmic material transfer between donor and host cells [114–116]. These studies stressed that the propensity for transplanted donor photoreceptors to integrate and provide functionality was more limited than initially thought, with many of the observed functional benefits likely coming from the donor-host transfer of phototransduction proteins [117,118]. The material transfer represents a novel therapeutic strategy for the rescue of diseased retinal cells [76,119]. However, for the evaluation of current photoreceptor transplantation experiments, the use of late-stage disease models in which the majority of photoreceptor cells are lost may be preferable for the assessment of cell integration and functional rescue. Nonetheless, the cell transplantation approach adopted, either cell suspension or cell sheet, may reflect the disease stage needing to be treated. In milder degenerative stages, donor cell suspensions may be advantageous. This is due to the preexisting architecture in which the donor cells can integrate into and support the remaining host photoreceptors. However, at later stages of degeneration, where few or no photoreceptors remain, retinal sheets may be more optimal at surviving and forming synaptic connections with the remaining inner retina.

Both photoreceptor cell suspensions and retinal sheets derived from ROs have been explored for the treatment of late-stage models of retinal disease in which nearly all host photoreceptors have already degenerated [62,108–110,120]. As an example, McLelland et al. used hESC-derived ROs as a source of healthy retinal tissue to rescue an immunodeficient late-stage retinal degenerative rat model. They found that the transplanted RO-derived retinal sheets went under differentiation and integration, leading to an improvement in visual function, despite the degenerative microenvironment. Analysis of the transplanted retinal sheets showed the presence of rod and cone photoreceptors with putative outer segments, bipolar cells, Müller glial cells, amacrine cells, and horizontal cells, which were all derived from the transplant. The presence of increased immunoreactivity for synaptophysin near the transplanted cells compared to areas further away from the transplant was suggestive of potential synaptic connectivity between the host and transplanted cells [109]. While the presence of transplanted matured photoreceptors with potential synaptic connectivity is extremely promising, the evaluation of tangible functional rescue in degenerative models is still limited. As an example, the immunodeficient rat model used both by McLelland et al. and Tu et al. were found to still have light-responsive patches at 10 months of age, making it difficult to

Table 1
Organoid models of retinal diseases.

Disease	Gene and mutation	Organoid features
LCA	CEP290 [90,91] c.2991 + 1655A > G homozygous mutation [90] Two patients heterozygous for IVS26 + 1655A > G and c.5668G > T mutations. One patient homozygous for IVS26 and 1655A > G [91] RPE65 [92] c.200T > G and c.430T > C	Aberrant splicing, impaired ciliogenesis, reduced cilia incidence [90] Abnormal ciliogenesis, docked mother centriole were only observed in optic cups derived from CEP290 LCA patients [91]
	AIPL1 [93] c.265 T > C homozygous mutation	All patient lines could generate well-layered ROs, which contained rods and cones that had inner segments and rudimentary outer segments. However, no photoreceptor phenotype was observed. In patient RPE cells, which were generated simultaneously with ROs, a significant decrease in RPE65 expression was found. No differences in biological function, as measured by POS phagocytosis and VEGF secretion, was found in patient iPSC derived RPE compared to control. Despite a reduction in both AIPL1 and PDE6B being found in patient ROs no retinal degeneration was detectable. Normal ultrastructural findings, expression of mature photoreceptor markers and similar gene expression profiles, except <i>NEUROD6</i> a transcription factor involved in amacrine cell subtype specification, were found between control and AIPL1-LCA ROs.
RP	RPGR [94] c.1685_1686delAT, c.2234_2235delGA, c.2403_2404delAG mutations CRB1 [72] Patient 1: c.3122T > C homozygous; Patient 2: 2983G > T and c.1892A > G; Patient 3: c.2843G > A and c.3122T > C USH2A [97] c.8559-2A > G and c.9127_9129delTCC	Significant defects in photoreceptors, including decreased photoreceptor cell number, cilia length, and expression of photoreceptor-related genes, were found between control and patient ROs. When the electrophysiological properties of patient rod-like cells were examined compared to controls they were found to have a reduction in hyperpolarization-activated potassium current. This along with expression data highlighted a deficit in HCN channels. Three hiPSC lines derived from <i>CRB1</i> RP patients, when differentiated into ROs, showed a phenotype of disruptions at the outer limiting membrane (OLM), as demonstrated by ectopic photoreceptor nuclei above the OLM. The degenerative retinal phenotype of <i>CRB1</i> RP patient hiPSC derived ROs was similar to that previously found in mice expressing variant <i>CRB1</i> ^{C249W} or lacking <i>CRB1</i> [95,96]. Significant defects were identified from an early stage in patient compared to control ROs including delayed retinal self-organization, abnormal retinal neuroepithelium differentiation, and defective retinal progenitor cell development. A reduced diameter and thickness of the retinal neuroepithelium was found along with an increase in expression of apoptosis related genes in patient ROs. Additionally, a decrease in retinal cells including photoreceptors, Müller and amacrine cell were found in patient ROs. Other RO defects included aberrant basement membrane and tight junctions. Further gene ontology analysis identified differentially expressed gene enriched for categories involved in calcium signaling, retinal layer formation, dopaminergic synapse and vesical transport.
Autosomal-dominant RP	PRPF31 [98] Three related RP type 11 patients with c.1115_1125del11 heterozygous mutation. One patient with severe RP with c.522_527 + 10 del heterozygous mutation.	Progressive degenerative features were found in ROs with TEM showing patient photoreceptors had an increase in apoptotic nuclei and the presence of stress vacuoles compared with controls. Additionally, a significantly reduced response was found in patient ROs to the neurotransmitter GABA. Patient ROs also exhibited impaired pre-mRNA splicing and had an increase in differentially expressed genes in gene ontology categories relating to the actin cytoskeleton, ciliary membrane, primary cilium, photoreceptor inner and outer segments, axon terminals and phototransduction. In accordance with this defective photoreceptor cilia were found in patient ROs.
Late-onset RP	PDE6B [65] c.694G > A homozygous mutation.	Patient ROs before DD180 exhibited relatively normal retinal development as compared with controls. However, patient ROs at DD230 were found to have defective rod cell migration. Furthermore, gene ontology analysis found an enrichment of gene implicated in G-protein-coupled receptor activity, G-protein-coupled receptor signaling pathway and calcium ion binding in patient ROs. Additionally, an increase in cGMP levels were found in patient ROs, which may have led to the impaired formation of synaptic connections and the decrease in photoreceptor cilia found in patient ROs.
X-linked retinitis pigmentosa (XLRP)	RP2 [99] R120X nonsense mutation c.358C > T	RP2 patient-derived ROs exhibit a spike in cell death in the ONL at DD150, with a subsequent thinning of the ONL detectable at DD180. The onset of cell death coincides with the timing of rod cell maturation and rhodopsin expression. In accordance with the thinning ONL, a reduction in the number of rhodopsin positive cells in the ONL was detected at DD180.
Glaucoma	OPTN [100] E50K missense mutation	Early stages of retinal differentiation in ROs were unaffected by the OPTN (EK50) mutation. However, at later stages of maturation, retinal ganglion cells from derived from patient iPSCs exhibited neurite retraction, increased excitability, dysfunctional autophagy, as marked LC3 accumulation, and an increase in apoptosis of the inner retinal organoid.
X-linked Juvenile Retinoschisis (XLRS)	RS1 [101] Patient 1: c.625C > T Patient 2: c.488G > A	Retinal splitting, outer-segment defects, abnormal paxillin turnover, defective retinoschisin production, impaired ER-Golgi transportation, defective photoreceptor connecting cilia, and altered expression of retinopathy-associated genes including IQCB1 and OPA1, which are associated with LCA and autosomal dominant optic atrophy, respectively).
Retinoblastoma	RB1 [103] RB1-null mutations [103]	RB1 was found to be abundant in retinal progenitor cells and become downregulated during maturation of ROs. Loss of RB1, in RB1-null ROs, promoted S-phase entry, lead to an increase in apoptosis and caused a reduction in the number of photoreceptors, bipolar, and ganglion cells. However, loss of RB1 was not sufficient to induce retinoblastoma formation in ROs.

LCA, Leber congenital amaurosis; CEP290, centrosomal protein 29; RP, retinitis pigmentosa; VEGF, vascular endothelial growth factor; AIPL1, Aryl hydrocarbon receptor-interacting protein-like 1; OS, outer segment; RO, retinal organoid; RPE, retinal pigment epithelium; RPC, retinal progenitor cell; cGMP, Cyclic guanosine monophosphate; ONL, outer nuclear layer; GPR161, G-protein-coupled receptor; OLM, outer limiting membrane; GFAP, Glial fibrillary acidic protein; OPTN, optineurin; NEUROD6, neuronal differentiation 6; PDE6B, Phosphodiesterase 6B; IQCB1, IQ Motif Containing B1; ERG, electroretinography; BM, basement membrane; HCN, hyperpolarization-activated cyclic nucleotide-gated; DD, differentiation day.

conclusively discriminate graft-originated responses from the remaining host activity [109,120]. This highlights the need for careful consideration when choosing which degenerative models to use for such experiments and also highlights the need to confirm functionality using numerous approaches.

The CRISPR/Cas9, a technique has been used to correct mutations in patient iPSC lines leading to the amelioration, when differentiated, of the RO phenotype compared to ROs derived from the uncorrected patient line. This has been successfully studied on RO models of RP and XLRs [94,98,101]. Patient corrected stem cell could be utilized as a source for autologous cell transplantation for the treatment of retinal dystrophies. Lastly, during RO differentiation, there is a concomitant generation of RPE. Dissected RPE spheroids from ROs or RPE spheroids generated from the remaining cell culture after RO sorting can be used to generate RPE monolayers or could be dissected to form RPE sheets [121]. Transplantation of RPE is a promising strategy for treating advanced stages of eye disease such as RP, AMD, and Stargardt disease (STGD1) in which significant RPE loss is found [122–124].

Applications of retinal organoids to gene therapy

Adeno-associated virus (AAV) is a small, nonenveloped, replication-deficient, single-stranded DNA parvovirus belonging to the genus Dependovirus. AAVs can be commandeered as a delivery vector for gene augmentation, currently the therapeutic strategy of choice for targeting hereditary retinal diseases [76,125]. Many studies have been performed using different promoter/serotype combinations to assess cell tropism and transduction efficiency of AAVs in ROs, with photoreceptors and radial glial progenitors/Müller glial cells being transduced [60,72,99,126–128]. Here, we provide two recent examples of AAV-mediated gene delivery in ROs.

Lane et al. used AAV2/5 to deliver the RP2 gene to photoreceptors in an organoid model of X-linked retinitis pigmentosa (XLRP). RP2 patient-derived ROs exhibit a spike in cell death in the ONL at differentiation day (DD)150, with a subsequent thinning of the ONL detectable at DD180. The onset of cell death coincides with the timing of rod cell maturation and rhodopsin expression. In accordance with the thinning ONL, a reduction in the number of rhodopsin positive cells in the ONL was detected at DD180. After AAV2/5. CAGp.RP2 gene therapy, an amelioration of this degenerative phenotype was observed, as marked by a reduction in ONL thinning and restoration of rhodopsin expression [99]. This study highlights that the therapeutic potential of AAV-mediated delivery of RP2 should be further explored as a treatment for XLRP.

The implementation of AAVs to deliver larger retinal genes is impeded by their limited cargo capacity of approximately 5 kb of DNA. Recently, Tornabene et al. used split inteins to mediate protein trans-splicing in human ROs. They found that multiple, AAV delivered, split intein-flanked polypeptides could be reconstituted to form large full-length proteins in mouse, pig, and human photoreceptors. Importantly, they were able to develop AAV-ABCA4 and AAV-CEP290 intein vectors to improve the retinal phenotype found in STGD1 and LCA type 10 (LCA10) mouse models, respectively. Additionally, they were able to find expression of ABCA4 in STGD1 patient-derived ROs after administration of AAV2/2-GRK1-ABCA4 intein vectors.

Patient-derived ROs are a promising alternative to animal models for the testing of AAV-mediated gene augmentation strategies. However, the variability in AAV transduction efficiencies in ROs needs to be addressed [60,72,99,126–128]. Variations in transduction efficiencies

may be due to the methodology used to generate ROs, promoter choice, viral titre, vector tropism, transgene detection method, timepoint of viral vector administration to RO, and the number of days post-administration before RO collection and analysis [99]. Particularly, the developmental timepoint specific bioavailability of receptors for AAV uptake may be a critical consideration for efficient transduction of RO cell types and therefore AAV vector choice [72,128]. Additionally, lentivirus is also capable of efficiently transducing photoreceptors in ROs and is an alternative delivery strategy to be further explored [85,106].

Retina chips

Numerous physiological processes and disease progressions in the retina involve the presence of cells and structures from multiple developmental origins. For instance, blood vessels and immune cells, originating from outside the retinal lineage, play a critical role in the onset and progression of diabetic retinopathy and the wet form of age-related macular degeneration (wet-AMD) [129]. Retinal organoids as previously described can reflect a majority of the cell types and architecture of the retina, but cannot faithfully recreate several important disease-relevant structures such as the inner and outer blood-retina barriers (iBRB and oBRB).

Organ chips are particularly well suited to model these tissue barriers since they enable a multilayered alignment of cells as well as a blood stream-like perfusion [130]. Thus, a prime application for retina-based chips is the modeling of the blood-retina-barriers (BRB), in particular, the outer BRB (oBRB), formed by retinal pigmented epithelial cells (RPE), and the adjacent choroidal microvascular network formed by microcapillary endothelial cells [131]. The simplest oBRB on-chip arrangement poses a two-channeled organ chip where RPE and endothelial cells are seeded on opposite sides of a porous membrane. Subsequent perfusion by a medium pump is then used to recreate a microvascular blood flow.

In 2017, Chen et al. applied this concept using the RPE cell line ARPE19 and human umbilical vein endothelial cells (HUVEC) [132]. The chip was used to study the migration of HUVEC cells into the RPE compartment in normal, hypoxic, and hypoglycemic conditions. In all conditions, a decline of RPE cell number was observed, whereas the HUVEC cells migrated through the membrane towards the RPE layer.

In the same year, Chung et al. presented a slightly different chip concept replacing the porous membrane by a fibrin hydrogel gap [133]. To mimic the three-dimensional choroidal vascular network, endothelial cells mixed with the fibrin gel were seeded in a channel below the fibrin gap. RPE (ARPE19) cells were seeded on the gel wall above in a separate chamber. Upon stimulation with the angiogenic growth factor VEGF, the choroidal endothelial cells infiltrated the fibrin gap and the RPE layer, recapitulating the pathogenic processes occurring in wet-AMD. Subsequent treatment with the anti-VEGF antibody bevacizumab, a widely used wet-AMD drug, could prevent the vessels spreading, emphasizing that blood-retina barrier chips can not only reproduce pathophysiological processes but also reproduce therapeutic approaches.

The next generation of chip systems was then aiming to not simply reproduce the outer barrier function of the retina but also to integrate the neural retina and the blood vessels barriers within the retina (iBRB). Following that idea, Yeste et al. used a multilayered chip design which was separated by several permeable membranes [134]. Besides the RPE (ARPE19) component, representing the oBRB and primary human retinal endothelial cells, representing the iBRB, they integrated a

neuroblastoma cell line (SH-SY5Y), mimicking the neural part of the retina. Via the integration of a transepithelial electrical resistance measurement (TEER) system, they then validated the barrier functionalities of the iBRB and oBRB.

The retina chip introduced in 2019 by Achberger et al. aimed for modeling the oBRB together with the neural retina [135] (Fig. 4). By combining hiPSC-ROs with adherently cultured hiPSC-derived retinal pigment epithelium (RPE), they combined all major retinal cell types on one platform. The benefits of this chip were to have a sub-RPE choroidal-like perfusion through the microfluidic channels of the chip, the recreate of the oBRB using iPSC-derived RPE and to connect the photoreceptors (PRC) of the ROs with their direct neighbor cell type *in vivo*, the RPE. This close PRC-RPE proximity led to an increase of photoreceptor outer segment number after 7 days of co-culture as well as active phagocytosis of photoreceptor segments by the hiPSC- RPE.

Applications of retina chips to drug studies

As retina chips are able to mimic physiological processes and barriers in the retina, they are of high interest for the preclinical evaluation of drug effects [135,136]. In a proof-of-principle study, the retina chip from Achberger et al. was supplemented with chloroquine (anti-malaria drug) and gentamicin (antibiotic drug), which have both a detrimental effect on the retina. The retinopathic and toxic side effects observed *in vivo* could be reproduced, shown by staining cell death by propidium iodide (PI) and the lysosomal marker LAMP2. High doses of chloroquine (80 mg/ml) resulted in significant PI staining and an increase in LAMP2, reflecting the lysosomal dysfunction observed *in vivo* [137]. The toxicity of Gentamicin in the retina chip was dependent upon the presence of RPE. A decrease in toxicity was found in the presence of RPE indicating that these cells might act as a barrier that protects the retinal organoids from gentamicin toxicity.

Retina chips have also been used to develop and test intraocular tamponades. Silicone oil (SO) is commonly used as intraocular tamponade in different eye diseases such as retinal detachment and giant retinal tears [138,139]. Chan et al. analyzed the formation of SO droplets in a microfluidic chip designed with a retinal ganglion cell line

to mimic the eye cavity [140]. In this study, the researchers could measure the size as well as the number of SO droplets and reproduced SO-aqueous interface.

Applications of retina chips to cell (transplantation) therapy

The efficiency of therapies to replace dysfunctional photoreceptors by RPCs is highly based on the migration of cells collectively and in the correct direction [141].

Mirsha et al. used a microfluidic model of the retina to study the effect of human and mouse eye geometries on retinal cell migration [142]. Using computational models and experiments, they found that a concentration gradient of stromal cell-derived factor 1 (SDF-1) was formed and played as a force for chemotaxis; in the high concentration of SDF-1, cells migrated toward higher concentrations of it while in low concentrations they did not move. Thakur et al. studied the adhesive and displacement dynamics of RPCs to check if the biomaterial used in transplantation can enhance and coordinate cell migrations [143]. Using a μ Lane, they found that cell-cell interactions are dominant when hyaluronic acid or laminin was used as a transplantation matrix while high cell-matrix interactions in fibronectin result in cell monolayer formation. These findings emphasize the importance of substrate nature in enhancing the efficiency of migration and consequently in the success of transplantation. The same concept was investigated further using *Drosophila melanogaster* as a model organism combined with microfluidics by Pena et al. [144]. A novel electro-chemotactic study of the migratory behavior of RPCs was conducted by Mishra et al. [145]. By fabricating a galvano-microfluidic system, they were able to establish tunable superimposed electric fields to study its effects on cellular motility. Interestingly, they found that by using a combination of electric field and SDF-1 concentration gradient, not only the migration distance can be increased about three times, but also the directionality will be enhanced. By performing bioinformatics analysis, it was found that the reason was the down-regulation of adhesion proteins simultaneously with the up-regulation of cytoskeletal regulatory proteins. These studies pave the way for developing more advanced RC models to simulate post-transplantation remodeling.

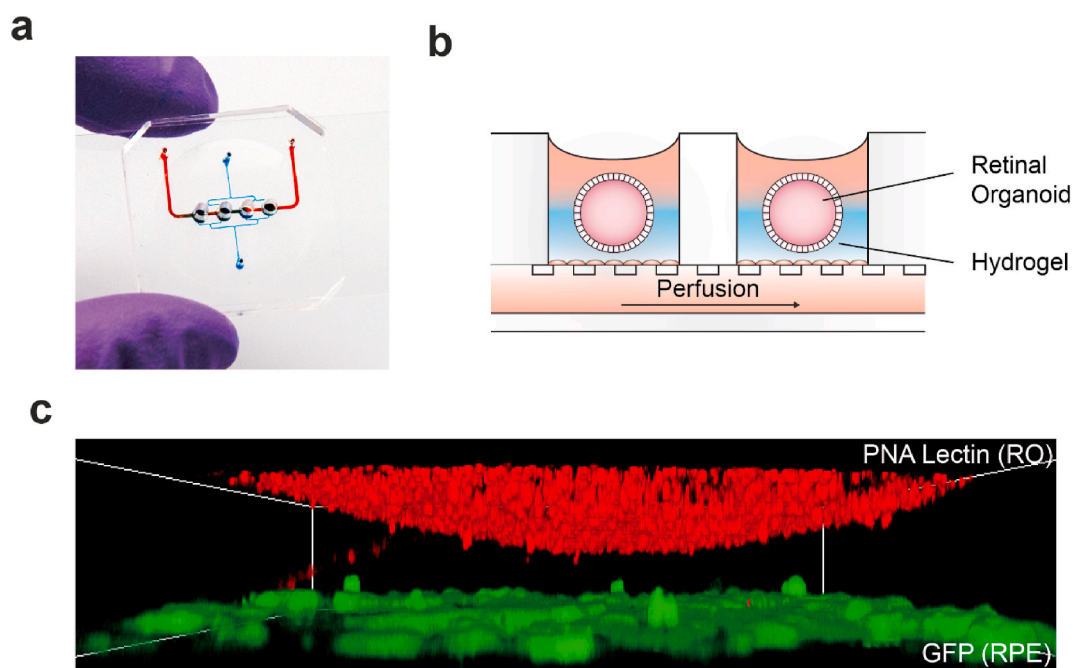


Fig. 4. The engineered retina chip model. (a) Image of the chip. Medium channels are colored with dyes for visualization. (b) A schematic concept of the retina chip. (c) A 3d reconstruction of a retinal organoid labeled by the photoreceptor surface marker PNA Lectin (red) and GFP-labeled hiPSC-derived RPE cells in the retina chip. Adapted from Achberger et al., 2019, CC BY 4.0 [135]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Lens

The initial steps for growing lens organoids or lentoid bodies (LBs) were made in the 1970s [146]. Three decades later, Yang and colleagues were the first to derive lens progenitor cells and LBs from hESCs [147]. They deployed a three-stage culturing system via sequential inhibition and activation of FGF, TGF- β , and Wnt signaling pathways. They could successfully culture a large number of LBs with this technique. Bone Morphogenetic Protein (BMP) signaling inhibition by noggin triggered cells towards neuroectodermal fate. Also, the role of FGF2 was found to be necessary for lentoid formation. Their model was limited in a few aspects. For example, terminal differentiation of the lens fibers was properly initiated but not completely executed, marked by the lower amounts of β - and γ -crystallins in LBs compared to the human lens but with comparable amounts in the initiation period. The LBs also tended to dissociate from their support from day 35 of culture. The use of 3D matrices containing laminin, collagen, and fibronectin, have allowed differentiation of LBs past day 35. Lacking light focusing ability was also one of the limitations of their model. Fu et al. used hiPSCs and induced them to lens progenitor cells and LBs by using a “fried egg” differentiation method. The cells in this method have a fried egg appearance at a time during differentiation, with the yolk serving as E-cadherin⁺ differentiating cells (D-cells) which eventually form the LBs, and the egg white serving as E-cadherin⁻ supporting cells (S cells) [148]. By using the same method of differentiation, they studied the autophagic activity in LBs derived from human iPSCs and ESCs. Their model can be used to investigate abnormalities in organelle degradation to form an organelle free zone (OFZ) during lens development [149]. Having an OFZ is crucial for the proper homeostasis of the lens, leading to cataract formation if disrupted. LBs have been successfully derived from cataract patient iPSCs [150]. The LBs can be used for studying various causes of cataracts, including congenital cataracts and age-related cataracts (ARC) [151,152]. For modeling ARC, it has been found that LBs spontaneously become cloudy after a prolonged time in culture, which is accompanied by protein aggregation. The use of hydrogen peroxide accelerates this process. This model system may be a suitable ARC disease model for understanding the role of oxidative stress in cataractogenesis [152].

Murphy et al. developed an alternative differentiation methodology that generated LBs which contained minimal non-lens cells [153]. In contrast to the previous LB models, their model yielded more spherical mini-lenses with a biconvex appearance and with more light focusing ability. They could purify their cells by performing Magnetic-activated cell sorting (MACS) to select cells expressing receptor tyrosine kinase-like orphan receptor 1 (ROR1), acting as a potential lens epithelial cell (LEC) purification agent. Their system is ROR1⁺ micro-lens system. They also suggested that their model can be used to study posterior capsule opacification (PCO), which is considered the most common complication of cataract surgery. Their model can effectively serve to understand the underlying mechanisms behind cataract risk factors, including age, diabetics, ultraviolet (UV) light, radiation, smoking, drug-induced, medications. The protein aggregates causing cataract or other particles causing light-scattering in the lens can be tested under high-throughput screening (HTS) conditions also for therapeutic targets. Most of the transcriptomic profile is expressed in LBs. Ali et al. compared the LBs derived from hESCs and peripheral blood mononuclear cell (pBMC)-originated hiPSCs in terms of their transcriptomic profile. They found that almost 70% of the total human protein-coding transcriptome is expressed in LBs. The hESCs and hiPSCs shared more than 96% in the similarity of transcriptome gene expression. However, they realized that the hiPSCs yield more differential gene expressions, including 416 down- and 291 up-regulated genes [154].

Discussion and conclusion

Engineered organoids and organ chips will inevitably change the way we do *in vitro* eye research in the future. In particular, they provide

us with the possibility to study individualized differences in disease manifestations [155]. Despite recent advances, there are some drawbacks and challenges that need to be addressed [156]. Technical variability is a major issue in organoid systems, as such standardized protocols are needed to avoid inconsistencies between results reported by different laboratories. Similarly, the use of well-characterized commercially available pluripotent stem cell lines would aid in the interpretation of results between labs. Lack of vasculature is preventing the study of vascular diseases like diabetes and high-risk corneal transplantation. Another important issue is the difficulty of modeling interactions between different eye tissues. Organ chips may help in this regard; however, this in turn comes with its own shortcomings [157]. Cornea and retina chips are powerful *in vitro* tools to study drug effects, therapeutic approaches, and disease-relevant questions, but the current chips, however, are still minimalistic. Future developments could make these chips even more complex, reflecting, and recreating an increasing number of functional and anatomical cues of the human eye, which may undergo critical changes in aging and disease [158,159].

We expect that future use of organoids and organ chips will lead to a surge of new research directions. Viral diseases and microbiome analysis are two areas where such approaches may impact ophthalmology. It is often hard to model human viral diseases in animals due to the specificities of virus-host interactions. Organ chips and organoids have recently been introduced to the field of virology [160]. Over the past decades, a major advance in medicine has been the recognition of the critical role of the microbiome in health and disease. The organ chip technology provides a way to study direct interaction between the microbiome and the different kinds of tissues outside the human body. For example, the intestine chip model provided a discovery tool to study the diverse microbiome-related therapeutics [161]. The significance of the ocular microbiome for ocular surface diseases such as DED has recently been recognized [162]. In the future, ocular microbiome chip technology will expectedly emerge as a new concept and an interdisciplinary approach in the pathobiology and clinical management of ocular surface diseases and beyond [162].

Finally, the full potential of *in vitro* modeling will only be realized if relevant measurement approaches are developed to extract biochemical and physical data from the engineered models. Advances in omics technology, in particular, are crucial in this regard. For example, metabolomics has only recently been introduced to the field of ocular surface diseases [163]. Application of these techniques to the human eye and the engineered models require protocols that allow for handling small volumes and a small number of cells. Current research in analytical biosciences in parallel to the bio-inspired engineering methodology promises great potential for organoids and organ chips in clinical translational research.

Lastly, organoids and organ chip technologies will allow for the testing of new therapeutic paradigms. For example, precision metabolome reprogramming was recently highlighted as a promising non-gene-specific therapeutic avenue for inherited retinal dystrophies which would be of interest to explore in a complex retina chip format which also contained microglia in addition to RPE and retina [164].

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Declaration of competing interest

None.

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