Advanced Drug Delivery Reviews 140 (2019) 33–50

Contents lists available at ScienceDirect

Advanced Drug Delivery Reviews

journal homepage: www.elsevier.com/locate/addr

Stem cell-based retina models

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ARTICLE INFO

Article history:
Received 19 December 2017
Received in revised form 16 March 2018
Accepted 12 May 2018
Available online 17 May 2018

Keywords:
Retinal organoids
Human iPSC
Optic cup
Optic vesicles
Retinal *in vitro* models
Drug testing
Disease modeling
Mechanistic studies
Translation

ABSTRACT

From the early days of cell biological research, the eye—especially the retina—has evoked broad interest among scientists. The retina has since been thoroughly investigated and numerous models have been exploited to shed light on its development, morphology, and function. Apart from various animal models and human clinical and anatomical research, stem cell-based models of animal and human cells of origin have entered the field, especially during the last decade. Despite the observation that the retina of different species comprises endogenous stem cells, most stem cell-related research in the human retina is now based on pluripotent stem cell models. Herein, systems of two-dimensional (2D) cultures and co-cultures of distinctly differentiated retinal subtypes revealed a variety of cellular aspects but have in many aspects been replaced by three-dimensional (3D) structures—the so-called retinal organoids. These organoids not only contain all major retinal cell subtypes compared to the physiological situation, but also show a distinct layering in close proximity to the *in vivo* morphology. Nevertheless, all these models have inherent advantages and disadvantages, which are expounded and summarized in this review. Finally, we discuss current application aspects of stem cell-based retina models and the specific promises they hold for the future.

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https://doi.org/10.1016/j.addr.2018.05.005
0169-409X/

1. Introduction

Vision is among the most important senses of the human neuronal system and loss of sight or blindness is considered as a major handicap in our society. Modeling the retina is therefore of common interest, concerning developmental studies as well as translational aspects. The visual system throughout the higher animal kingdom is based on a comparable cellular composition conciliating light-induced signal generation and conduction mode into the nervous system. The retina itself is a highly organized system of primary neuronal sensor cells, modifying interneurons and neurons projecting to the central nervous system (CNS). The complexity of these five major neuronal subtypes—photoreceptor cells (PRCs), horizontal cells (HCs), bipolar cells (BPCs), amacrine cells (ACs), and ganglion cells (GCs)—has been discovered throughout the last few decades, even demonstrating a large variety within the mentioned subtypes (reviewed in [1]). The retina is already processing the conducted light information, which involves contrast modification, light adoption, or spatial compression, for example [2]. These mechanisms are crucial for the specificity of sight as the number of PRCs greatly exceeds the capacity of the optic nerve; the information is therefore already compressed and adapted before reaching the conscious and unconscious receiver modules of the CNS [3]. Developmental studies have already been conducted in highly diverse model systems comprising vertebrate and non-vertebrate organisms since the early 20th century [4]. These investigations have successfully elucidated various cellular mechanisms and signaling pathways in drosophila, chicken, or Xenopus, for example, while *in vivo* studies on molecular biology and development in mammals have been widely performed in mice. Consequently, most *in vitro* studies were performed with mouse cells. For a long time, the human system remained unexplored in many aspects; knowledge of the human retina was based on studies in patients, rare retinal biopsies, and tissue from deceased people. Additionally, questions regarding human retinal development were rather difficult to answer. These limitations were challenging to overcome as the retina differs among species not only in its organization and morphology but also regarding developmental cues and protein distribution or function. A human model system mimicking both development and function was strongly required. With the discovery of human pluripotent stem cells (PSCs), a new field of research around the retina opened up and was soon adopted as a valuable model system. In sum, stem cells of endogenous and exogenous sources represent a great hope for *in vitro* developmental studies, disease modeling, and translational aspects such as cell replacement or the stimulation of endogenous stem cell pools *in vivo*. Here, the human retina is one of the most promising organ systems eligible for modern cell-based therapies.

2. A short glance at retinal development

In vertebrates, the retina is derived from the neuroectoderm—or more precisely, from the anterior neural tube [5]. There, the first retinal progenitors are gathered in a single eye-field as part of the diencephalon [6]. During the final phases of neural tube formation, this area provides the basis for two bilaterally evaginating structures—the optic vesicles. The progenitors found herein are positive for the early retinal markers visual system homeobox 2 (VSX2), also called ceh-10 homeodomain-containing homolog (CHX10) and microphthalmia-associated transcription factor (MITF) [7]. Subsequently, the distal part of the vesicle acquires contact with the invaginating lens anlage from the surface ectoderm and—mostly due to high fibroblast growth factor (FGF) release—loses its MITF expression [7,8]. Thereafter, the CHX10+ MITF− cells specialize into neural retina progenitors while the dorsal population (still expressing MITF) gives rise to the retinal pigment epithelium (RPE) [7]. Next, the apical neural retina invaginates, bringing itself into close apposition to the distal RPE (see also Fig. 1). The arising "cup" structure is thereafter called the "optic cup". Over an extended period, the neural retina progenitor cells reside and give rise to all retinal neurons and the one major glia type of the retina—the Müller cells. The order and number of the terminally differentiated neurons and glial cells are highly conserved [9,10]. The "early-born" neurons appear first, including GCs, HCs, ACs, and cone photoreceptors, whereas the "late-born" retinal neurons include rods, BPCs, and Müller glia (also reviewed in [11]) (see Fig. 1). The further sub-specialization of photoreceptors to rods and, in case of humans, to three cone types (long-wave, middle-wave, and short-wave) is influenced by a number of crucial transcription factors such as cone-rod homeobox (CRX), neural retina leucine zipper (NRL), orthodenticle homeobox 2 (OTX2), and thyroid hormone receptor beta 2 (TRβ2) [12–16]. A schematic overview of the retinal development is given in Fig. 1.

The *in vitro* differentiation strategies discussed here are mostly based on the idea of mimicking embryonic development by making use of similar signaling cues and molecules as present *in vivo*. The turns and intermediate steps, however, are largely dependent on the starting cell type. The next section gives an overview of the cell types and differentiation strategies applied in the past, including endogenous retinal cells, adult/fetal multipotent stem cells, and, most recently, PSCs.

3. Sources for retina models

3.1. Adult/multipotent stem cells

The first efforts in investigating the regenerative potential of the retina can be dated back >100 years ago, with experiments, mainly performed in amphibians, showing their ability to replace lens and retina [17–20]. Over time, many different animal model systems have been analyzed in this regard; only since the identification of neurogenesis in the adult human brain [21–26], however, was the hypothesis postulated that the adult retina of humans, as part of the CNS, could also harbor some kind of neural stem cells (NSCs) or progenitor cells [27]. Later, even potential cell sources from outside the retina, lacking any neural origin, were identified, including hematopoietic, mesenchymal, cord blood, and amniotic membrane stem cells, for example (reviewed in [28]).

This section provides an overview of the identified endogenous retinal stem cell types from inside the retina of various species as well as the stem cells from outside the retina, their applications as retina model systems and therapeutic vehicles, and the advantages and disadvantages of these model systems (see also Table 1). A graphical summary of the cell sources for retinal *in vitro* models can be found in Fig. 2.

3.1.1. Retina models from endogenous stem cells in the retina

The types of endogenous stem cells identified and verified in the retina comprise Müller glia, ciliary pigment epithelial cells, and retinal pigment epithelial cells (reviewed in [29]) showing the potential of

proliferation and, to some extent, multi-lineage differentiation. Nevertheless, regeneration of the retina in vertebrates is a very inhomogeneous phenomenon across species; the potential of endogenous stem cells underlies a strong inter-species discrepancy (Fig. 2A).

3.1.1.1. Müller glia. Müller cells are the major glia cell type of the retina. They span all retinal layers, they establish contact with neurons in different layers, and their processes form the inner and outer limiting membrane (reviewed in [30]). Therefore, this cell type not only fulfills structural functions but also acts as a selective barrier and is involved in retinal homeostasis [30]. Most interestingly, the Müller glia cell is also the retinal cell type studied in greatest depth for its stem cell characteristics.

Within the retina of fish and bird, replacement of degenerated cells was identified as a vital aspect of Müller cell function [27,31]. Especially in teleost fish such as *Danio rerio* (zebrafish), Müller glia retain the full ability to dedifferentiate, proliferate, and subsequently re-differentiate into neuronal subtypes and glia following trauma or exogenous growth factors [27,31]. The Müller glia also shows expression of retinal progenitor markers such as paired box gene 6 (Pax6) at low levels; consequently, these cells can be assumed to harbor multipotent stem cell potential in these species [31]. Furthermore, in rodents as well, the gene expression profile of Müller glia is comparable to that of retinal progenitor cells [32–34]. The possibility of *in vitro* maintenance of Müller glia derived from rat retina and their ability to reenter the cell cycle were already shown in very early studies [35,36]. It should be

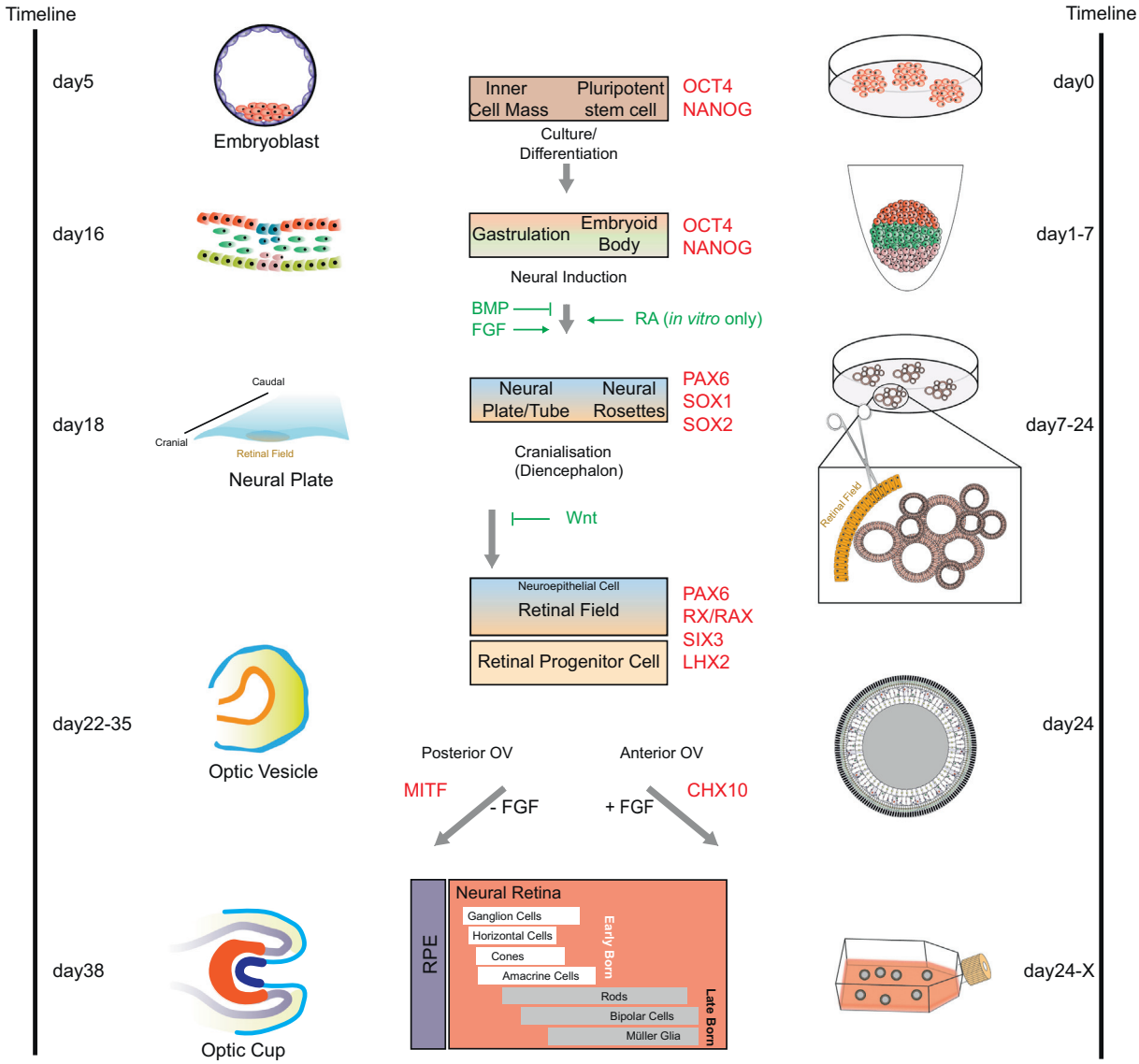

Fig. 1. Overview and comparison of human *in vivo* and *in vitro* retinogenesis. During embryonic development, a subset of the pluripotent stem cells from the embryoblast, or inner cell mass, will acquire a neuroectodermal fate. Neural induction in the embryo occurs *via* formation of the neural plate containing neuroepithelial cells. In the medial anterior neural plate, a single eye field develops and eventually evaginates to form bilateral optic vesicles. The optic vesicles contain retinal progenitor cells, which are the precursors of all neural retina cell types as well as of pigment epithelium cells. The anterior portion of the vesicle develops into the neural retina while the posterior part will form the RPE. The neural retina finally invaginates to form a cup-like structure, the "optic cup". Stages of development during retinal embryogenesis are shown on the left, the corresponding stages during retinal organoid differentiation are shown on the right, with their respective timeframes given. The middle panel provides information on the pathways that are involved in each stage of differentiation (in green), as well as the most important stage-specific marker genes (in red). Normal arrows with arrow head indicate activation, arrows with blunt end indicate inhibition of the respective developmental steps. Early and late born retinal cell types that originate from retinal progenitor cells are shown at the bottom (scheme adapted from Bassett et al., 2012 [11]). Abbreviations: BMP, bone morphogenetic protein; CHX10, ceh-10 homeodomain-containing homolog (VSX2); FGF, fibroblast growth factor; LHX2, LIM homeobox 2; MITF, microphthalmia-associated transcription factor; NANOG, Nanog homeobox; OCT4, octamer-binding transcription factor 4 (POU5F1); OV, optic vesicle; PAX6, paired box gene 6; RA, retinoic acid; RPE, retinal pigment epithelium; RX/RAX, retinal homeobox; SIX3, SIX homeobox 3; SOX1, SRY-box 1; SOX2, SRY-box 2.

noted, however, that the process of Müller glia transdifferentiation is far more limited in mammals after retinal injury or PRC loss [37].

However, since primary Müller cells are difficult to obtain and, in most cases, result in cultures of low purity with a limited lifespan, immortalized human and murine Müller cell lines were developed and analyzed for their stem cell properties [38]. Under the appropriate neural culture conditions, the spontaneously immortalized human Müller cell line MIO-M1 showed neural stem cell characteristics such as expression of tubulin beta 3 class III (TUBB3), SRY-box 2 (SOX2), PAX6, CHX10, and notch homolog 1 (NOTCH 1) as well as retinal neuron markers like brain-specific homeobox/POU domain protein 3 (BRN3), S-opsin, or recoverin [39]. When transplanted into the subretinal space of rats, migration into the PRC, inner nuclear, and GC layer was observed as well as further differentiation toward the respective local retinal cell types [39].

Using defined factors, human Müller glia can be differentiated directly into mature retinal cell types *in vitro*, such as retinal GC precursors and PRCs shown by the respective marker profiles [40–43]. Moreover, transplantation into the inner retinal surface of a degenerative retinal rat model led to partially restored visual function [40,41]. Supplementation with taurine, retinoic acid, FGF2, and insulin like growth factor 1 (IGF1), on the other hand, resulted in a highly efficient differentiation of PRCs [42,43]. Also in this case, transplantation into degenerated retinae of rats resulted in integration into the outer nuclear layer and improvement of photoreceptor-associated visual function [43].

The identification of the stem cell-like characteristics of Müller glia has put a new perspective on this retinal cell in the context of basic and clinical research. So far, much hope remains regarding the use of Müller glia for the regeneration of the retina. They exhibit a broad regenerative potential *in vitro* and in non-human transplantation settings. Nevertheless, applicability for clinical trials in humans is still hindered, most probably due to the lack of understanding of human Müller glia physiology. Additionally, in the human retina, the regenerative potential of Müller glia seems to be unable to administer and activate self-repair after injury- or disease-related degeneration.

3.1.1.2. Retinal pigment epithelial cells. Another cell type of the retina—the pigmented layer of cells called RPE—has been found to possess

Table 1
Summary and evaluation of available retina stem cell models and their applications.

			Multipotent Stem Cells		Pluripotent Stem Cells		
			Eye & Retina	From Outside the Retina	Adherent Culture	Optic Vesicle/Optic Cup	RPE
Features	Accessibility		Human cell lines available (Müller, RPE) (reviewed in [28]) Human primary cells limited	Variable availablitiy	Easy to obtain from every individual	Easy to obtain from every individual	Easy to obtain from every individual
	Cell Types Derived in vitro		RPC, GC, PRC, RPE, Müller glia [32-34; 39-43; 54-56; 223] Not all retinal cell types	PRC, PBC, RPE, RPC, GC [70; 71; 78-86] Not all retinal cell types	RPC, PRC, GC; RPE [97; 105-109; 189] No extra-retinal cells, no full subtype diversity reported	PRC, all retinal neurons, RPE, Müller glia [94-96; 100-101; 129-133; 135] No extra-retinal cells, no full subtype diversity reported	RPE [104; 138-144]
	Retinal Layering in vitro		Microfluidic device [223]	No complex studies	No complex layering	Advanced Embryonic [94; 96; 101; 131; 160; 161]	-
	Synaptic Wiring in vitro		Microfluidic device [223]	No complex studies	No complex layering	Synapses of OPL, IPL [131; 135] No advanced complexity, no outer plexiform triad synapse	-
	Functionality in vitro		PRC electrical stimulation, phototransduction markers expressed [42; 43] GC show Ca2+ responsiveness [41]	Light sensitive PRC [79-81] electrophysiolocially active GC [86]	Phototransduction markers expressed [97] No full functionality	Light sensitive [101; 174] No retinal processing reported yet	Phagocytosis, homeostastis [135-138; 140]
	PRC Morphology in vitro		Simple (bipolar morphology) [42-43] No full maturity	Simple (segment-like structure can be detected) [71] No full maturity	No full maturity, functionality	Advanced (IS and OS)[101; 131] No full maturity of OS	-
Application	Transplantation	Current Status	Studies performed in mouse/rat, human [39-43; 199] (reviewed in [200; 201])	Multiple studies, different adult stem cells [202] (reviewed in [196; 200; 201])	Adherent PRC transplanted in mouse [204]	Transplantation of PRC or retinal sheets in mouse [161]	Studies performed in humans [205]
		Drawbacks	Long term improvement, reproducibility	Long term improvement, reproducibility	-	Central nervous system connection to the brain, No human studies yet	-
	Disease Modeling	Current Status	No complex studies	No complex studies	Studies performed (e.g. retinitis pigmentosa, Usher syndrome, Glaucoma) [187-189]	Studies performed (e.g. Leber congenital amaurosis, glaucoma) [133; 191]	Studies performed (e.g. Best disease) [129; 190; 194]
		Drawbacks	-	-	Surrounding structures missing	Surrounding structures missing; late-onset disorders hard to mimic	Only RPE, effects on NR are not modeled
	Developmental studies	Current Status	Regneration and transdifferentiation analyzed in animals [31; 45-53; 57; 58]	No complex studies	Studies on retinogenesis [157]	Studies on degeneration, regeneration, retinogenesis [95; 96; 183]	Studies on retinogenesis [95]
		Drawbacks	Limited in mammals [37; 50-53] artificial differentiation process	-	-	Surrounding structures missing	Only RPE, effects on NR are not modeled
	Drug Testing	Current Status	Proof of principle [223]	No complex studies	Possible [187]	Possible [163]	Possible [129; 194]
		Drawbacks	Purity, complexity not given so far	No complex systems	Surrounding structures missing	Complex readout, lacking immune cells, not fully mature, lacking vasculaization	Only RPE, effects on NR are not modeled

Green = Advantages
Red = Limitations

regenerative stem cell properties and therefore might harbor the ability to differentiate into other retinal cell types. *In vivo*, the RPE forms part of the blood-retinal barrier and is responsible for nutrient supply of PRCs, absorption of scattered light, phagocytosis after disc shedding of outer segments, and supplying the retina with essential neurotrophic factors to maintain its integrity (for review refer to [44]).

The regenerative potential of RPE cells was first discovered in amphibians such as tadpoles and urodeles and in chicken embryos, where cellular transdifferentiation of RPE cells into retinal neural progenitors was observed [45–48] (reviewed in [49]). Remarkably, even after the depletion of the entire neural retina, RPE cells in these animals can dedifferentiate, lose their pigmentation, detach from the Bruch's

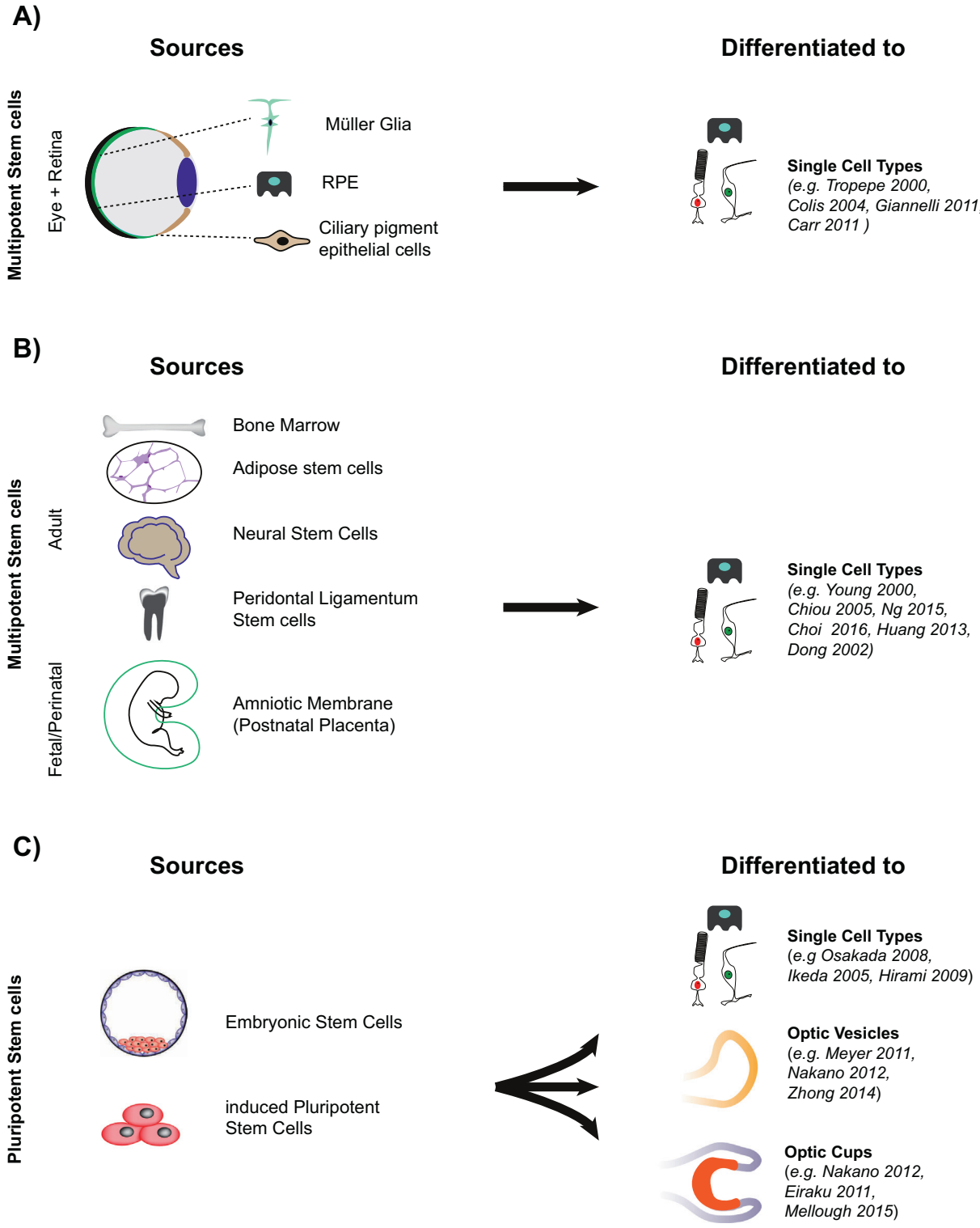

Fig. 2. Sources for retinal *in vitro* models. Overview of starting material for *in vitro* retinal differentiation (left) and resulting differentiated cell types (right). Multipotent stem cell sources include retinal (A) and extra-retinal cell types (B). Those stem cell sources have limited capacity and can be differentiated into single retinal cell types. (C) Pluripotent stem cell sources include embryonic and induced pluripotent stem cells. They have the capacity to differentiate into all neural retina cell types as well as into RPE. Existing protocols can derive either single cell types, optic vesicles or optic cups from pluripotent stem cell sources. Abbreviations: GC, ganglion cells; PRC, photoreceptor cells; RPE, retinal pigment epithelium.

membrane, start proliferating, and eventually form a completely new retina possessing all layers with normal connectivity [47]. This ability of the RPE is lost after only a few days in chick embryos, while it remains active throughout life in amphibians [46,48].

In mammals, again, only the very early embryonic-stage cells seem to retain a certain degree of stem cell characteristics in their RPE; for example, rat embryonic RPE was shown to be able to transdifferentiate *in vitro* into neural retina cells in the presence of basic fibroblast growth factor (bFGF) [50]. However, in adult mammals, a proliferative potential is kept *in vivo* and is enhanced after retinal damage [51,52], but the potential to transdifferentiate seems to be lost [53]. Consequently, the ability to enter the cell cycle is still present in humans as well; hence, the RPE represents an attractive tool to model retinal development *in vitro* if the necessary regulatory elements to control transdifferentiation are activated. In line with this, treatment of the immortalized adult RPE cell line-19 (ARPE-19) and of primary human RPE explants with the retinoic acid derivate fanretinide can direct the cells toward a retinal progenitor-like cell identity [54]. In 2012, another study investigated human RPE stem cells (RPESCs), which harbor the potential to generate RPE and even show neural and mesenchymal differentiation potential [55]. Moreover, this study revealed that suspension conditions promote stemness in the multipotent RPE, whereas in adhesion culture, cell growth and division are preferred [55].

Another quite successful approach involved the direct reprogramming of human RPE cell lines ARPE-19 and telomerase reverse transcriptase (hTERT)-immortalized RPE cell line hTERT-RPE1 using overexpression of neuronal differentiation 1 (NEUROD), neurogenin 1 (NEUROG1), or neurogenin 3 (NEUROG3) [56]. The derived photoreceptor-like cells expressed retinol binding protein 3 (IRBP), recoverin, retinal cone arrestin 3 (ARR3), transducin alpha-subunit, CRX, and a cone opsin [56].

These studies showed the ease of accessibility of RPE-derived multipotent stem cells, and their potential as a model system for not only RPE generation but also other retinal cell types. This could be useful for cell-replacement therapies and disease modeling. Nevertheless, the full potential of the derived cell types has not yet been investigated in detail, and the question arises whether mature retinal cell types can also be derived without the forced overexpression of neuronal factors.

3.1.1.3. Ciliary pigment epithelial cells. The ciliary marginal zone (CMZ)—also called circumferential germinal zone or peripheral growth zone—is found at the periphery of the neural retina in fish, frogs, and birds, connecting it to the ciliary body. In the mentioned species, this region contains retinal stem and progenitor cells that can differentiate into new retinal neurons, mainly during development but also during regeneration [47,57,58] (review in [59]).

In mammals, the existence of a CMZ is controversial; however, isolated cells from the adjacent pigmented ciliary epithelium can be propagated *in vitro* and were identified as neural progenitor cells with retina-specific expression of Chx10 [60,61]. Although these cells represent a mitotic quiescent population under normal circumstances, with the addition of exogenous growth factors *in vitro*, they can be expanded and express stem cell markers; in some studies, they also exhibited the potential to differentiate into early- and late-born retinal neurons [62]. Hence, a great overlap of the cellular and molecular profile of ciliary pigment epithelium cells and retinal progenitor cells has been described. Other studies, on the other hand, were unable to confirm this differentiation potential and therefore postulated that—despite showing self-renewal and expression of certain neuronal marker—ciliary pigment epithelium cells still retain markers of mature cells, do not lose their pigmentation, and consequently should not be regarded as retinal progenitors or stem cells [63].

In summary, it has not yet been clarified whether the described cell type really shows a full stem cell profile and would be suitable as a retinal model system to obtain a fully functional and physiological human retina. Nevertheless, cells from the ciliary epithelium might still be interesting for unveiling some of the molecular requirements of retinal regeneration and research conducted in this direction should not be neglected.

3.1.2. Stem cells from outside the retina

The ability of fetal/adult multipotent stem cells to form tissues or cell types *in vivo* or *ex vivo* is strictly limited and, in most cases, specific to the tissue of origin. However, in the last few decades, numerous studies have led to a new paradigm—that stem cells of different origins can give rise to not only progeny of their natural potential but also unrelated cell types, through an artificially induced transdifferentiation (reviewed in [64]). This process comprises two steps: First, the cell loses its specific characteristics and expression hallmarks, leading to a less-differentiated state. This dedifferentiation process is naturally present in mainly non-mammalian animals as a mechanism of regeneration, *e.g.* in the myocardic or lens tissue [65–67]. In the second step, an embryonic differentiation program is initiated, allowing the cell to take a new fate. Since the state of differentiation can be affected and controlled by the cellular niche and environment (reviewed in [68]), the first experiments involved transplantation into animal retinae. In that course, NSCs and bone marrow stem cells were applied [69,70]. In case of NSCs, few calbindin-positive cells could be detected four weeks after transplantation into the rat retina, but no cells were positive for PRC markers [69]. Transplanted bone marrow cells showed expression of rhodopsin, calbindin, and other neural markers, albeit not to a great extent [70]. Human bone marrow stem cells were subsequently cultured with human RPE cells *in vitro* [71]. Under these conditions, co-cultured and differentiating stem cells started to express protein kinase C (PKC) which is a BPC marker and opsin as PRC marker. In addition, some cells developed a longish process appearing as a segment-like structure [71]. In conclusion, the transplantation and co-culture of adult stem cells were at least partially successful in generating cells expressing retinal markers. However, the approach did not generate mature retinal cells in high numbers or purity and the value as an *in vitro* model may be limited.

A second idea of inducing transdifferentiation in adult stem cells is based on the forced overexpression of distinct transcription factors. During embryogenesis, the presence and function of certain retinal transcription factors are crucial, and their depletion leads to the loss of the correlating cell type or cell function [72–74]. Therefore, it was assumed that the overexpression of these factors could induce cell identity. In a similar fashion, this has been shown, among others, for cortical neurons using neurogenin 2 (NEUROG2), overexpression [75] or myogenic differentiation (MyoD) overexpression [76] for inducing a muscle-cell fate.

As one of the earliest markers of the neuroepithelium and the eye field [77], PAX6 was overexpressed in a study from 2014 [78]. Here, the isoform 5a from PAX6 was used to transdifferentiate adipose-derived stem cells—a recently discovered type of adult stem cells. Five days after induction, the expression of the photoreceptor markers CRX (64% of the cells) and rhodopsin (44%) was detectable. Moreover, 33% of the cells expressed the RPE marker retinoid isomerohydrolase (RPE65). Other crucial markers for retinal, and especially photoreceptor, differentiation, CRX, retinal homeobox (RX), and NEUROD, were employed in two studies from 2012 [79] and 2014 [80]. As a starting cell type, iris pigmented epithelium cells (IPE) and fibroblasts were selected. After transfection, rhodopsin, blue opsin, red/green opsin, and other PRC markers could be detected. The results, however, were variable, depending on the combination of the used transcription factors [79,80]. Astonishingly, the arising photoreceptors showed light response, demonstrating an advanced degree of functionality [79,80].

In a very recent study from 2016, PRC differentiation was induced in peripheral blood mononuclear cells (PBMCs) by CRX overexpression alone. Similar results could be obtained including retinal and PRC marker expression as well as light responsiveness [81]. Two studies from 2015 and 2016 went a step further by influencing a whole network

of transcription factors *via* inhibiting miRNAs in amniotic stem cells (AESCs) [82,83]. With this approach, they were able to generate RPE (miRNA410) [82] and PRCs (miRNA 203) [83] with the respective marker expression.

In 2013, a study investigated periodontal ligamental stem cells (PDLSC), which showed neurogenic differentiation potential under appropriate conditions [84,85]. By using neural induction medium in combination with the bone morphogenetic protein 4 (BMP4) antagonist Noggin and dickkopf WNT signaling pathway inhibitor 1 (DKK1), it was possible to induce the expression of two early retinal markers PAX6 and RX and rod photoreceptor markers [84]. With minor modifications, it was also possible to generate BRN3B- and NEUROD1-positive retinal ganglion-like cells that were electro-physiologically active [86].

In summary, stem cells from within and outside the retina have been used to generate cells with properties of retinal neurons. Most of the studies, however, could only show scattered marker expression, producing either a certain retinal neuron type or an arbitrary mixture. In general, the approaches lacked most of the physiological retinal features, such as synaptic wiring, layering, or the occurrence of elaborated light-sensitive photoreceptors (Fig. 2B).

3.2. Embryonic stem cells and induced pluripotent stem cells

PSCs harbor the amazing abilities to differentiate into all cells of an organism, together with an indefinite division potential. Since the first successful isolation and cultivation of mouse [87,88] (1981) and human (1998) embryonic stem cells (ESCs) [89], these cells are utilized to create virtually all tissues and cell types *in vitro*. However, in most countries, the use of human ESCs remains strictly limited since their generation requires the destruction of an embryo, raising strong ethical issues and discussions. Moreover, these cells cannot be generated from any individual; if used for transplantation, they would in most cases provoke HLA-based immune rejection (reviewed in [90]).

The thrilling discovery of induced pluripotent stem cells (iPSCs) in 2006 by Takahashi and Yamanaka [91] generated a way out for most of these problems. They discovered that these stem cells could be created artificially from almost any somatic cell of the body and harbor the same characteristics as ESCs, including unlimited proliferation, expression of pluripotency network genes, and the ability to differentiate into cells from all three germ layers. The fact that these cells can be generated from every person makes them extremely suitable for (stem-) cell replacement strategies and especially for *in vitro* disease modeling.

Since then, ESCs and iPSCs have been used to create an increasing number of tissues and cell types of all three germ layers. Among them are numerous of neuronal cell types such as dopaminergic neurons [92], hippocampal neurons [93], and retinal neurons [94–101] (see Fig. 2C). Notably, the used protocols underline the fact that the same differentiational cues that are responsible for the embryonic development of a tissue or cell type, including retinal neurons, can be applied for *in vitro* differentiation (reviewed in [102]) (Fig. 1). As discussed above, for retinal cells, this includes (i) the neural induction of the PSC and (ii) a neural patterning into the direction of the anterior neural plate—more specifically, of the diencephalon where the retinal field is located [102].

3.2.1. Adherent culture models

One of the first experiments aiming to generate retinal cells made use of stromal cells from bone marrow (PA6 cells) as feeder cells in order to induce neural identity in mouse embryonic stem cells (mESCs). The cultures originally gave rise to mostly midbrain dopaminergic neurons [103,104], but variations of the protocol *via* the addition of retinoic acid, cholera-toxin, and dexamethasone enabled some cells to acquire a neural retina, lens, or RPE cell fate [105].

In the search for more defined ways to induce neural cells, Zhao et al. [106] used retinoic acid and serum-free conditions in presence of insulin, transferrin, selenium, and fibronectin for the neural induction of mESCs. The protocol ended up in a high percentage of Pax6 and nestin-positive cells. Subsequent co-culture with PN1 primary retinal cells resulted in cells positive for several markers of retinal neurons; a small proportion of the cells became positive for mature photoreceptor markers such as rhodopsin. However, none of the positively stained PRCs could acquire any morphological or functional specialization such as segment or process formation.

In 2005, Ikeda et al. applied a method for neural induction in mESCs based on the generation of floating embryoid bodies, which were kept under serum-free conditions (Serum-free Floating Embryoid Body-like [SFEB] aggregates) [107]. This made the cells susceptible to Wnt (Dkk1) and Nodal-antagonizing factors (left-right determination factor 2, LeftyA). Both factors effectively induced neural differentiation in 90% of the cells, especially toward a rostral neural phenotype (mainly mediated by the Wnt antagonist Dkk1). By overexpressing the photoreceptor factor Crx in retinal progenitors, they successfully induced rhodopsin expression in >20% of the infected cells. Similar effects were reached on co-culturing RPCs with dissociated embryonic retinal tissue [107]. A good step forward was achieved with purified mouse ESC-derived Rx-positive cells using a knock-in Gfp strategy and the SFEB protocol [97]. The selected population was reseeded and treated with the Notch inhibitor (N-[N-(3,5-difluorophenacetyl)-l-alanyl]-*S*-phenylglycine t-butyl ester) DAPT, known to induce the photoreceptor lineage [108]. After 20 days, >20% of the cells showed PRC-marker expression (Crx), with a minority showing expression of rhodopsin or cone opsins. This could be increased *via* the addition of a combination of acidic fibroblast growth factor (aFGF), bFGF, taurine, sonic hedgehog (Shh), and retinoic acid at a later stage of maturation. In the same fashion, by using SFEB followed by an adherent culture state and the late addition of retinoic acid and taurine, they were able to induce photoreceptor differentiation in monkey and human ESCs [97]. In both species, opsin- and rhodopsin-positive populations could be identified after 130 to 200 days. Although these cells showed expression of various functional photo-transduction genes such as transducin, phosphodiesterase, and Arr3 among others, these cells still lacked proper morphology or functionality [97]. In 2009, comparable results were achieved using human and mouse iPSCs [109].

In summary, the endeavor to create adherent retinal cells was successful in the sense that markers for several cell types were identified. Nevertheless, these protocols lacked a proper retinal organization, a concerted arising of all cell types and, most important, functionality. However, the methods used for 2D cultures led to the discovery of the self-organizing nature of suspension-cultured retinal progenitors and thus, the emergence of laminated retinal organoids.

3.2.2. Three-dimensional organoid culture

The basis for 3D *in vitro* models for non-mammalian and mammalian cells was established over a century ago in dissociated and reaggregating marine sponge cells [110]. The mechanism behind the ability of cells to self-adhere and self-organize—forming free-floating cell spheroids—is strongly linked to the naturally occurring embryonic processes.

The first breakthrough in stem cell associated 3D formation was the generation of cortical structures from mouse ESCs in suspension culture [111]. This was probably a milestone report, clearly demonstrating the ability of differentiating stem cells to recapitulate tissue and organ development in the dish. Most interestingly, the generated polarized neural tissue development occurred without gross extrinsic signaling pathway modulation. Therefore, it can be assumed that the cellular program required during embryogenesis is present in each cell, together with the distinct cell-cell interaction cues.

In the recent decade, numerous groups have reported the generation of a broad variety of 3D self-organizing organ-like structures from mouse and human stem cells; finally, the term “organoid” has become commonly accepted in the scientific community. Organoids are defined as 3D cellular structures exhibiting function and morphology close to

the respective *in vivo* counterpart (reviewed in [29,112–115]. While morphological criteria include the cellular layout and polarization of each particular cell in the tissue-specific laminate, functional criteria cover the production of tissue-specific factors or signal conduction in the layered cell network (*e.g.* neuronal signal conduction).

The value and usability of human-PSC-derived complex organoids has already been proved for numerous tissues from all germ layers, including pancreas [116,117], liver [118], gut [118], lung [119,120], kidney [121–123] or brain [111,124], as well as for the retina.

3.2.3. Retinal organoids generated from pluripotent stem cells

In the previous sections, we discussed the recent approaches investigating two-dimensional (2D) cell models, mostly comprising single-cell subtypes of human and animal origin. Although a variety of developmental and clinical questions could be answered by exploiting these culture systems, the physiological system of the highly organized retina and its cellular interactions were impossible to mimic adequately (see also Fig. 1). With the first description of 3D retinal structures derived from PSCs, the tide had turned. The initial study by Meyer et al. from 2009 showed not only the initiation of eye-field precursor differentiation from human PSCs, but also retinal progenitor clusters exhibiting marker expression representative of early retina formation in 3D [95]. Their protocol was based on the observation that ESCs and iPSCs are prone to take anterior neuroepithelial characteristics when cultured serum-free and without exposure to any modulating factors other than proneural proliferation medium based on the B27/N2 supplement formulation [125–128]. Although the 3D clusters could give rise to photoreceptor-like cells displaying CRX and opsin expression, a more complete retinal organoid formation was missing. The Sasai group first succeeded in clearly demonstrating the spontaneous generation of stratified optic-cup-like structures in 3D floating mouse ESC differentiation cultures [94]. Almost simultaneous to their study with mouse ESCs, the first report of a more physiological human optic vesicle-like organoid was published [129]. Their observations of a stratified retina structure emerging autonomously from PSCs opened new venues for retinal research and started a wave of subsequent investigations, setting up more and more precise protocols and providing advanced characterizations. It should be noted that both classes of PSCs—ESCs and iPSCs—were successfully and comparably used for the generation of human retinal organoids [130,131]. Interestingly, probably the most widely accepted published protocols for the generation of human retinal organoids differed broadly in their differentiation approaches. The first of these protocols, by Nakano et al. in 2012, relied on the extrinsic modulation of several pathways including Wnt, SHH, and FGF signaling, directing PSCs to a neurogenic fate with a subsequent ventralized determination and optic-cup-like formation [96]. Further, the formation is held out completely in 3D with the optic vesicles initially growing out of the EB. In 2014, Zhong et al. countered this paradigm by clearly demonstrating that retinal organoid and optic-vesicle formation is achievable in a nearly self-directed manner in adherent culture followed by selected detachment of areas with retinal field identity [101]. Nevertheless, in both approaches, retinal portions need to be morphologically defined; early retinal structures are excised and subsequently cultured to generate pure retinal structures. Assuming that general steps of maturation occur autonomously, and subsequent final maturation is conducted physiologically *via* intrinsic factor release, extrinsic modulations may be reduced to a minimum. Intriguingly, Zhong et al. were the first to show the initial maturation of photoreceptor outer-segment formation—a key structure that had been missing in retinal organoids until then. Moreover, they could show a certain electric response upon light exposure—a crucial functional evidence.

In the last few years, several groups have tried to improve the yield of certain cell types and structures within the retinal organoids. For example, the Notch inhibitor DAPT in the early/middle stages of retinal differentiation was used to increase the proportion of photoreceptors within the laminated organoids [100,101,131]. Other publications tried to increase the yield of GCs and combined the 3D organoid differentiation with subsequent 2D culture [132,133]. The resulting neurons showed increased ganglion marker expression as well as directed outgrowth of axon/optic nerve-like structures [132].

Other improvements focused on the increase in retinal cell yield, *e.g.* the implementation of hydrogel cultures. Hunt et al. [134] showed that the use of hyaluronic acid or alginate-based hydrogels can increase the yield of retinal organoids and the expression of neural retina and RPE marker expression. In another report, the addition of IGF1 to optic-cup structures increased the lifetime of optic cups and enabled accelerated photoreceptor maturation [135]. Although their protocol was also the first to show a direct interaction of photoreceptor and RPE, the maturation of photoreceptors was not yet comparable to optic vesicle differentiation. Overall, retinal organoids—namely optic cups and optic vesicle cells—are the most recent, most advanced, and probably the most physiological *in vitro*-formed retinal structures, with formation of neural retina and RPE. However, since RPE can be generated in an independent and more distinct way, we discuss its differentiation methods in a separate section.

3.2.4. RPE derived from pluripotent stem cells

As mentioned before, RPE within the retina is vital for the survival of photoreceptors. In disorders like age-related macular degeneration (AMD), retinitis pigmentosa, or diabetic retinopathy, the RPE can be primarily affected (pathology of retinal degeneration reviewed in [136]). This in turn leads to the degeneration of the PRCs, as they are no longer adequately supplied (reviewed in [137]). Consequently, the generation of RPE from PSCs with the goal to model the pathology of or restore vision in the mentioned disorders is of great interest.

The first successful differentiation of stem cells into RPE was achieved rather coincidentally as a by-product in a study using ESCs from primates, originally intended to obtain dopaminergic neurons [104]. PA6 mouse stromal cells were used to induce neural differentiation in a method known as stromal cell-derived inducing activity (SDIA) [104]. Primate ESCs already showed areas of pigmented cells after three weeks of differentiation. Moreover, the typical RPE cobblestone-like morphology could be observed; the cells stained positively for Pax6 and could be isolated and cultured without feeder cells [104]. Subsequent studies using the same method of differentiation also showed expression of RPE markers such as zonula occludens 1 (ZO-1), RPE65, retinaldehyde binding protein 1 (CRALBP), and MER proto-oncogene, tyrosine kinase (MERTK) [138]. Functional tests proved their ability to phagocytose latex beads; when transplanted into the subretinal space of rats, the differentiated RPE cells improved photoreceptor survival [138].

The same basic and simple differentiation method can also be applied to human ESCs. Differentiation of human ESCs into RPE can be achieved in co-culture with mouse embryonic fibroblasts in a period of four to eight weeks. Feeder-free or embryoid body-based differentiation is also applicable and has been shown to be even faster (three to four weeks) compared to the culture on feeder cells [139]. The process of RPE differentiation in this context was described as “an inevitable event,” since all embryoid bodies eventually became pigmented, if given enough time [139]. The human ESC-derived RPE expressed the relevant marker genes of developing and mature RPE (OTX1/2, PAX6, premelanosome protein PMEL17, RPE65), proving its cell identity and close proximity to a primary human RPE-expression profile. Their functionality was shown in phagocytosis-assays and transplantations [139–142].

Even though the described undirected protocols reliably generate RPE, the efficiency was still rather low. Consequently, protocols were established to achieve a more directed and faster differentiation with higher yields of RPE cells. Directed culture protocols included, for example, the addition of nicotinamide [143] to generate RPE within four weeks. This effect could even be augmented by the addition of the transforming growth factor-beta (TGFβ)-signaling molecule activin A,

which was shown to direct optic vesicle differentiation into RPE [143,144]. Further, other pathways were also shown to be involved, as the inhibition of NODAL by their respective antagonists DKK1 and LEFTYA led to a higher efficiency of RPE generation [97].

Also for iPSCs, the differentiation can be performed in a spontaneous [145] or in a directed manner [109] using the same factors as mentioned above for the ESC-derived RPE. Additionally, several other proteins or small molecules were described that promote RPE differentiation, including the small molecules CKI-7 and SB431542 to inhibit WNT and NODAL signaling [98]. In contrast, the activation of canonical/b-catenin WNT signaling with CHIR99021 improved RPE derivation efficiency at a later time-point of differentiation; consequently, the stage of differentiation seemed to play an important role for the effect of WNT activation *vs*. inhibition [146]. In a similar fashion, the inhibition of FGF signaling with the factor SU5402 seemed to be effective [95]. The known RPE-inducing factors were further combined with previously described general retina-inducing factors like NOGGIN, DKK1, bFGF, IGF1 [147,148], retinoic acid, and SHH [148,149] at defined time-points.

Protocols based on 3D suspension cultures, as described in the 3D organoid section, are also suitable for generating RPE cells [95,99,101,150]. As opposed to the generation of optic vesicles, areas of retinal progenitors are not detached for suspension culture but kept as adherent culture to directly generate RPE with high efficiency [95,99]. However, even if directed RPE generation is not intended, the suspension culture will to some extent also lead to RPE cluster generation [101].

On the other hand, the protocol published by Nakano et al. [96] focused on a balanced expression of neural retina and RPE as a prerequisite for optic-cup formation. In a specific timeframe, WNT signaling was activated here with CHIR99021 to increase MITF expression. Subsequently, an invagination and formation of optic cups could be observed, and therefore a correct morphology and layering of the RPE and its position toward the neural retina.

To summarize, the directed protocols to generate RPE at high purity are of relevance, especially for transplantation settings such as a treatment option for AMD, where RPE is selectively degenerated. However, to investigate a physiological retina "in a dish," the role and interplay of RPE in optic vesicles is extremely interesting for the development of functional model systems.

4. Features of current retinal *in vitro* models

The retina is able to conduct a series of complex events aiming for the transition of light quanta into chemical and subsequently into electrical information. Furthermore, the light information translated by the photoreceptors is strongly condensed, interpreted, and preprocessed by a series of horizontal and vertical information transfers [3]. The cells that are responsible for these tasks are the retinal neurons, building a complex network of electrical and chemical synapses. Consequently, a truly physiological model needs to recapitulate this functionality. Most of the promised applications of *in vitro* models rely on the fact that they can mimic some or all of the features of the retina *in vivo*. Therefore, recent stem cell models need to be critically evaluated in terms of their ability to reproduce these crucial retinal characteristics. An overview of the features of iPSC-derived retinal organoids is provided in Fig. 3.

4.1. Cell type diversity

A central prerequisite for the complex processes performed by the mammalian retina is the presence of various retinal cell types. Following embryonic development, retinal cells can be categorized by their origin—cells that derive from a common progenitor within the retinal field, comprise six types of retinal neurons, the retinal pigmented epithelial cells, and one type of glial cells—the Müller glia [151]. In contrast, all blood vessels, endothelial cells, and immune cells such as microglia are derived from mesodermal and yolk sac mesodermal origin and infiltrate the retina during development or postnatally [152–155]. Moreover, astrocytes and oligodendrocytes presumably populate the retina and the optic nerve *via* migration from other parts of the CNS [156].

The 2D and 3D models described in the last section exhibit a varying potential to generate these cell types. Most of the initial adherent stem cell-based protocols aimed for the generation of retinal progenitors or single-cell types such as photoreceptors, although some studies already showed a mixture of generated retinal neurons [97,105,107,147,157]. The reports also lacked the presence of retinal Müller glia. Here, the retinal organoids and 3D-based protocols show one of their major advantages—they harbor all major retinal cell types, including Müller glial cells, and all retinal neuron types, namely PRCs, HCs, ACs, BPCs, and retinal GCs [96,101]. A crucial aspect of the mammalian retinal make-up is the photoreceptor diversity. In humans, one type of rod and three different kinds of cones (short-, middle, and longwave sensitive) can be distinguished. Already, the initial retinal organoid reports could identify these subtypes by their specific chromophores (for rhodopsin and opsin) [96]. Even more astonishingly, they could be microscopically separated by their unique rod and cone morphology. Recent publications have also revealed the presence of specific subtypes for ACs [158].

In contrast to the complex retinal organoids, the generation of RPE was already established in adherent 2D cultures [104]. Unlike retinal neurons, the pigmented epithelial cells could be efficiently generated on coated plastic dishes presenting morphological features such as pigmentation, a cobblestone-like morphology, and expression of RPE-specific markers [139]. In retinal organoids, pigmented epithelia are also frequently found. Their orientation largely depends on the generation mode: In the Eiraku and Nakano protocol resulting in optic cups, RPE is found in close apposition to the apical neural retina, where the photoreceptors would be located [94,96]. In protocols where retinal fields are lifted from adherent culture and subsequently formed in suspension, the RPE is fully displaced and has no clear relationship to the neural retina [101]. Nevertheless, these cells also show a strong pigmentation and RPE morphology; when reseeded, they give rise to an RPE layer similar to that in 2D-based protocols [96].

Finally, it must be stated that a physiological retina would require the presence of blood vessels, endothelial cells, immune cells, and CNS astroglia. Recent approaches using self-organized organoid spheres are based on single-origin progenitors and therefore lack the ability to generate cells from more than one germ layer or place of origin [159].

4.2. Morphological cues

The mammalian retina exhibits a highly organized architecture concerning order and orientation of the retinal cell types. The basis for this organization is already set up during early development and the time course of the arising of different cell types is well-preserved. When Nakano, Eiraku, and others found that the *in vitro* self-organization of organoids follows the same spatial and temporal cues and terms of embryonic development, it was not surprising that they could also give rise to an advanced form of layering [94,96,160]. In several reports, the organoid's ability to form different retinal layers could be observed, including inner and outer limiting membranes (ILM, OLM) [96,161], the innermost GC layer, an inner plexiform layer containing AC, BC, HC, and glial cell soma, and an outer nuclear layer exclusively containing cell soma of rods and cones [101,131]. Finally, the photoreceptors form segments that are located above the outer limiting membrane growing out of the organoid. Electron microscopy imaging revealed a complex arrangement of inner and outer segments connected by primary cilia [101,131]. Comparable to the *in vivo* situation, the inner segments are rich in mitochondria while the outer segments present premature forms of disk stack formation [101,131]. In mouse-derived PSCs, segments were found after appx. 25–35 days [94]. In the human-PSC system, however, the process of segment formation takes

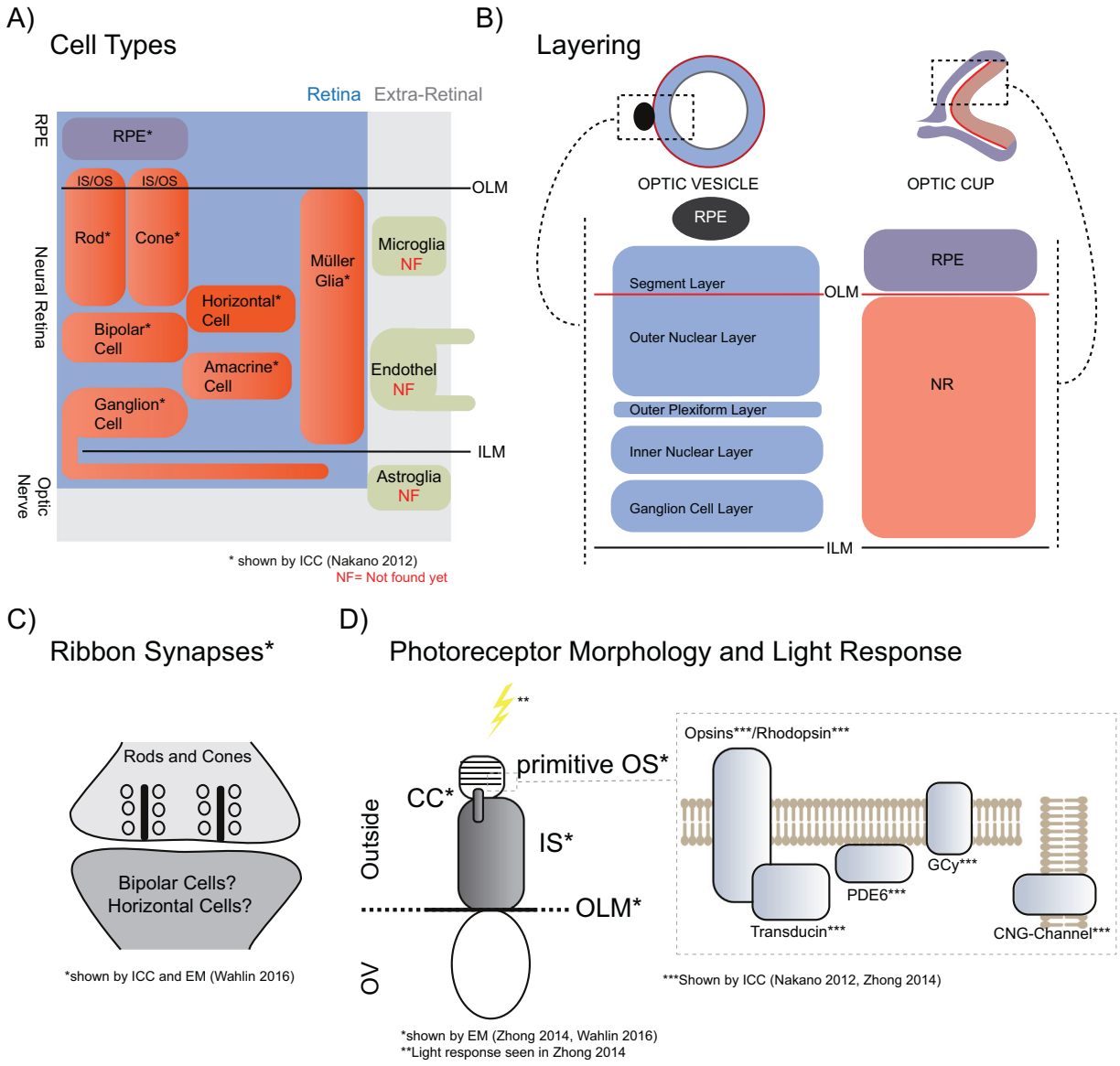

Fig. 3. Features of iPSC-derived retinal organoids. A) Organoids harbor all retinal neurons and the major retinal glia cell type, the Müller cells. Cells which integrate from extra-retinal tissues, such as the CNS and mesoderm, are not found. B) iPSC-derived optic vesicles display an advanced form of layering with an outer and inner nuclear layer separated by a distinct synaptic outer plexiform layer. A separated ganglion cell layer in the center of the organoid can be found. Arising RPE clusters have no direct contact to photoreceptors. Inner and outer limiting membranes are present. Optic cups are less advanced in layering but show a clear apposition of RPE and neural retina. C) Outer plexiform synapses display ultrastructural presence of ribbons inside the photoreceptor end feed and synapse formation. Postsynaptic cells could be horizontal or bipolar cells. D) Light response *via* chemical transformation into an electric signal is a main feature of photoreceptor cells. Photoreceptor cells in organoids *in vitro* display a premature/primitive outer segment, express all required proteins for phototransduction and respond to light stimulation [101]. Abbreviations: CNG-channel, cyclic nucleotide-gated ion channels; EM, electron microscopy; GCy, guanylate cyclase; ICC, immunocytochemistry; ILM, inner limiting membrane; IS, inner segments; NR, neural retina; OLM, outer limiting membrane; OS, outer segments; OV, optic vesicles; PDE, phosphodiesterase; RPE, retinal pigment epithelium.

a multiple of the time (120–200 days) following species and size differences [96].

The interaction of photoreceptors and RPE—which is important for nourishing and creating a microenvironment for the outer segments *in vivo* (reviewed in [162]), is not established in optic-vesicle-like organoid cultures as a consequence of the displaced RPE clusters [101]. In optic cups, as first described by Nakano and Eiraku, this interaction was established in an early fashion; however, the structures were not stable over a long period [94,96,163]. A recent study faced the problem of unstable and imperfect optic-cup maturation by a specific differentiation protocol including IGF1. As a result, these cultures were stable for >90 days, including photoreceptor differentiation and synapse formation. Unlike the optic vesicle, these structures showed a directed apposition of photoreceptors and RPE and therefore more closely resembled the physiological situation [135].

4.3. Synaptic wiring

An essential function of the retina is its ability to conduct and process light information detected by the photoreceptors to downstream neurons performing horizontal and vertical preprocessing. To a great extent, this is implemented by the two gatherings of synapses in the inner and outer plexiform layers [164]. The outer synapses, constructed by photoreceptors to interconnect with BPCs and HCs, is permanently active in the state of light inactivation [165]. Consequently, the photoreceptor pre-synapses harbor highly specialized structures called ribbons, enabling continuous calcium-based vacuole production and exocytosis (reviewed in [166]). Recently, it has been shown that these ribbons can also be found in organoid photoreceptor synapses [131]. Immunostainings and electron microscopy showed that the photoreceptors indeed form this specialized synapse and consequently might

be able to conduct signals to BPCs [131]. Complex gatherings, such as the outer plexiform triad synapse [167,168] constructed by HCs, photoreceptors, and BPCs, have not yet been identified in retinal organoids.

The inner synaptic layer containing the connections between BPCs, ACs, and GCs is so far inadequately studied in stem cell-derived organoids, most probably because the inner layer and especially the GCs disappear after a certain culture period [100,101]. Since BPCs form late in the development of the embryo [169], they cannot be found *in vitro* before the GCs disappear. Nevertheless, early forms of synapses have been found in the inner layer of IGF1-treated optic cups, probably forming between GCs and other neurons of the inner nuclear such as ACs [135].

4.4. Light sensitivity

The most intriguing feature of a retinal organoid is its ability to respond to light stimuli. In mammals, light sensation is recorded and conducted in the photoreceptor's outer segment, where the chromophores rhodopsin and L/M/S- opsins binding 11-cis retinal can change their conformation and enzymatic activity of transducin upon photon absorption [170–172] (see also Fig. 3D). Following a downstream cascade, the signal is amplified, leading to a hyperpolarization of the cell's membrane potential and finally to a glutamate decrease at the synapse [173]. The necessary proteins and structures (inner and outer segments of the photoreceptors) have been identified in iPSC-derived retinal organoids [101,131]. In human retinal organoids, rhodopsin and opsins can be found starting from around day 120 and at day 190, while the first light responses were monitored at around 190 days of differentiation *via* patch-clamp [101]. Although the number of active cells was still low, and the measured signals were quite weak, these experiments proved that the requirements for a proper light response of the photoreceptors are given. The reason for the yet small signals might be found in the still immature outer-segment structures, the fragile nature of the suspension culture conditions, and the non-physiological extracellular environment. One way of increasing photo-sensation was suggested in a study by Busskamp et al. in 2014 [174]. By overexpression of the miRNA cluster 182, 96, 183 in mouse-ESC-derived retinal organoids, they largely accelerated the development of outer segments; subsequently, a high percentage of light-sensitive PRCs could be observed.

4.5. Current drawbacks

Despite the presented features, retinal organoids are still far away from the highly complex construction of the mammalian retina *in vivo*. Some of the missing features are shared by all *in vitro* organoid systems, such as the missing vascularization, smooth muscle cells, or immune cells like microglia [159,175,176]. Therefore, it would be necessary to equip the organoids with endothelial cells, as has been achieved for liver organoids, for instance [118]. In addition, the mesoderm-derived microglia, which are involved in retinal immune responses, have not yet been implemented in retinal organoids. A promising approach would be the external addition of these cells, originating from primary or PSC sources.

The fact that organoid development closely follows embryonic development is accompanied by unfavorable properties. The human development and maturation of the retina, which take around eight to nine months, are finished postnatally [177]; similar time spans were observed for PRC maturation *in vitro* [96,101,131]. This makes human retinal organoid culture time consuming and more prone to general degeneration processes.

Another major issue is caused by the 3D nature of the optic vesicle organoid. Although the mammalian retina is a plane sheet *in vivo*, the organoid is built as a spheroid with equal distribution of the retinal tissue on the outside. This is most likely because the sphere-form is the favorable state of cell populations in suspension [178,179]. The lacking orientation surrounding tissues and extracellular matrix prohibits polarized tissue development, which is necessary for optic-cup development and peripheral-central specialization. Further, the nutrition of the innermost layers is only possible through passive diffusion and therefore limits the organoid growth and viability. It might also cause the frequently observed degeneration of the inner cells, most prominently the GCs, which disappear before the photoreceptors in the organoid can fully mature [100,101].

The developmental stage of the organoid retina can be compared to a late fetal stage, with established synapses and cell types. Nevertheless, the adult *in vivo* retina exhibits a far more complicated intercellular connectivity compared to the *in vitro* system and comprises a huge variety of cell subtypes in the major cell categories (reviewed in [180]). Moreover, the *in vivo* retina is made of a complex and conserved arrangement of rods and cones and their consecutive connection to variable cues of on- or off-triggers [181,182]. This complexity of stratification and arrangement has not yet been reached in the *in vitro*-generated organoids by far.

Moreover, photoreceptors in retinal organoids from either mice or humans still exhibit immature outer segments—a prerequisite for physiological and exact function. *In vivo*, the trafficking of outer segments and proper nutrition of PRCs are mainly performed by cells of the RPE (an overview is given in [162]). In that respect, the complementation of matured retinal organoids with a functional layer of RPE cells might provide a more physiological system for a more physiological and mature retina *in vitro* (see also Table 1).

5. Application of retinal *in vitro* models

Independent of the tissue-type, 3D-stratified and functional organoids derived from PSCs provide a *bona fide* platform to shed light on human cell and tissue development—a prerequisite for translational aspects. PSC-derived studies represent herein a unique system to follow the entire development, starting from early embryonic events over fetal and finally peri- or postnatal situations.

In this respect, retinal organoids closely mimic the physiological retina *in vivo* concerning layering, cell subtype presence, and functional connectivity, have already emerged as an exciting system not only to investigate the developmental aspects of the human retina, but also to represent a well-defined platform for disease modeling and pharmacological application design. Here, we briefly discuss the most exciting fields of application for stem cell-derived retinal cells, the obstacles that still need to be overcome, and especially, the future opportunities they offer (see also Table 2).

5.1. Mechanistic studies

Organoids, as a tool and model system, may give essential and fundamental insights into developmental and regenerative processes. However, before being able to generate organoids resembling “organs in a dish” with all their physiological and functional properties, one must closely mimic the *in vivo* development of the respective organ or tissue.

In this regard, studies on degeneration and regeneration can be conducted in organoids that have emerged as a novel research platform and may, at least in part, replace animal experiments [163].

Further, several studies investigating retinal organoids were executed with the goal to reveal basic principles of retinal development and the role of individual genes and proteins within this process. This includes genes involved in early retinogenesis and the generation of retinal fields. The significance and expression patterns of genes like PAX6, a known master regulator for retinogenesis, have been analyzed using retinal organoids, for example with the help of transgenic hPAX6GFP reporters [183]. In other cases, genetically modified reporter cell lines like RX and CRX-Venus fluorescent reporters have been used to monitor their expression during the course of retinal differentiation [96]. The

Table 2
Summarized information obtained on retinal organoid formation, features, improvements and limitations.

Retinal Organoid (RO) formation method	Description	Source
Combination of 2- & 3-dimensional RO formation	Adherent undirected neuronal differentiation	Zhong et al. 2014 [101]
	Selection of eye field areas and self-organization of RO in suspension	Meyer et al. 2011 [129]
3-dimensional RO formation	Serum-free floating EB formation (SFEB)	Eiraku et al. 2011 [94]
	Ventralization by WNT inhibition	Nakano et al. 2012 [96]
	Outgrowth of RO positive spheres	Mellough et al. 2015 [135]
3-dimensional optic cup formation	Serum-free floating EB formation (SFEB)	Eiraku et al. 2011 [94]
	Ventralization by WNT inhibition	Nakano et al. 2012 [96]
	Balance of RPE and NR differentiation lead to OC formation	
Features/characteristics	Description	Source
Retinal cell types	Details see Fig. 3	Zhong et al. 2014 [101]
Retinal layering		Meyer et al. 2011 [129]
Synapse formation		Eiraku et al. 2011 [94]
PRC maturation		Nakano et al. 2012 [96]
PRC light sensitivity		Wahlin et al. 2017 [131]
Improvements	Effect	Source
Addition of notch-inhibitor (DAPT)	Increase PRC number and maturation	Nakano et al. 2012 [96] Zhong et al. 2014 [101]
Addition of retinoic acid, fetal bovine serum (FBS) and taurine	Increase RO size, survival and differentiation	Zhong et al. 2014 [101]
Alginate hydrogel encapsulation	Increase of RO yield	Hunt et al. 2017 [134]
Addition of IGF1	Enhance optic cup differentiation	Mellough et al. 2015 [135]
	Acceleration of PRC maturation	
Adherent ganglion cell culture & addition of BDNF	Enlarged ganglion cell population forming functional axons	Tanaka et al. 2015 [132]
		Ohlemacher et al. 2016 [133]
Limitations	Description	Source
Time consuming	RO maturation in human >200 days	Zhong et al. 2014 [101] Wahlin et al. 2017 [131]
Central tissue degeneration	Nutrition and oxygen supply by diffusion limits organoid size	Reviewed in Yin et al. 2016 [159]
	Leads to central organoid malnutrition and degeneration	
Missing vascularization	Absence of blood vessels, endothelial cells and pericytes	Reviewed in Yin et al. 2016 [159]
Missing immune cells	Absence of microglia	
Central GC and AC degeneration	GC disappear in ROs >100 days	Zhong et al. 2014 [101]
Imperfect PRC outer segment formation	Rare, immature and unorganized OS	Nakano et al. 2012 [96] Zhong et al. 2014 [101] Wahlin et al. 2017 [131]
Missing RPE-PRC interaction	RPE not in close interaction with mature PRC segments	Zhong et al. 2014 [101] Wahlin et al. 2017 [131]
Retinal subtype diversity	Identification of retinal subtypes difficult (no markers available/no morphological discriminability) *e.g.* ON/OFF pathways	

role of the key neural retina transcription factor CHX10 has been analyzed using stem cells from a microphthalmia patient with a selective mutation in the respective gene [184].

Another option to investigate the function of single genes is genetic modification in the stem cell, or even at later differentiation stages, by directly switching on or off the expression or using total gene disruption. In this context, gene-editing using CRISPR/Cas9 is a promising approach for a rapid mutation and screen of gene function; it is already being applied to the organoids of other types of tissue [185].

In summary, studies investigating human retinal differentiation at very early stages of development have gained exciting new possibilities with the use of retinal organoids. The role and effect of key regulator genes in this context can be analyzed with patient-derived cells as well as with targeted genetic modification of single genes of interest.

5.2. Disease modeling

Most diseases and syndromes involving the impairment and degeneration of the retina finally lead to blindness and are incurable as of now. The successful treatment demands a thorough understanding of the underlying pathomechanisms; consequently, a variety of disease models exists. In the past, animal models such canines, cats, chicken, pigs and especially rodents have been used to reflect certain aspects of the retina with associated pathogenic mechanisms and have led to a greater understanding of many crucial aspects (review on retinitis pigmentosa in [186]). However, human eyes are in many aspects unique—for example concerning their macula densa or their sophisticated trichromacy. Therefore, not all diseases and drug responses might be fully reflected in animal models. Consequently, there is a need for new systems that can truly reflect the human genetic background as well as its cellular and anatomic makeup.

In this respect, the use of *in vitro* cell models and especially of retinal organoids has provided us with new insight in human pathology. Human iPSCs as a source, enable the generation and investigation of patient-specific cells, which harbor the exact genetic makeup, including the genetic background and not merely the distinct mutation. In the last few years, an increasing number of studies have dealt with the generation of patient-specific iPSCs and the unraveling of the pathomechanisms of degenerative disease like retinitis pigmentosa [187], Usher syndrome [188], normal tension glaucoma [189] and Best disease [190] among others. An extraordinarily promising study was performed in 2016 by Parfitt et al., who were able to define and rescue the cilia-related disease mechanism caused by a mutation in the CEP290 gene in a 3D patient-specific retinal-organoid surrounding [191]. This study showed the value of iPSC-derived retinal cells at present and possibly in the future.

Although the iPSC-based *in vitro* retinal models offer great opportunities for identifying key players and pathways of many diseases, several factors concerning the suitability of this model system have to be considered. First, the modeled disease requires the corresponding structures. For example, disorders involving the immune system, or the endothelium of the choroid might not be truly reflected since these structures or cells are still lacking in iPSC-derived organoids. Further, the time of disease onset needs to be considered. Disease manifestations that happen late in life, *e.g.* in late-onset retinitis pigmentosa, might be hard to analyze in the rather short-termed culture duration.

In summary, when used properly, stem cell-based retina models can be beneficial for gaining insights into many disease aspects. This

includes situations where animal models have been shown to be unable to faithfully mimic human diseases of the retina. Although animal trials of new therapeutic approaches may not be circumvented by the exploitation of human retinal organoids, this system could at least reduce the use of animals in pre-clinical investigations.

5.3. Drug testing and safety assessment

The advancement of generating individualized retinal cells from patient-derived human iPSCs also offers exciting new possibilities regarding personalized drug treatment. The patient-specific effects of different pharmaceuticals can be tested on the patient's own retinal organoids or RPE to find the best individual treatment option. Further, retinal organoids also provide a new possibility of testing pharmaceuticals, whether or not they are intended as treatment for retinal disorders. The development of any new pharmaceutical agent requires the analysis and testing of its pharmacodynamics and should also include the testing of adverse side-effects in the eye.

Surprisingly, there is a wide range of systemic drugs that can have ocular side effects, usually if applied as long-term treatment. The list includes pharmaceuticals like corticosteroids, which are used for a variety of allergic conditions, antibiotic therapies for tuberculosis like rifabutin and rifampin, the anti-malaria drugs chloroquine and hydroxychloroquine, and tamoxifen, which is used in breast cancer treatment (reviewed in [192]). Some of these drugs have already been used on retinal organoids or RPE in culture to mimic the side-effects and find indications of the molecular pathways involved in the pathology. Administration of tamoxifen can potentially lead to retinal toxicity, including macular edema, peripheral crystallin deposits, and vision loss [193]. Treatment of mouse iPSC-derived retinal organoids with 4-hydroxytamxifen was shown to results in photoreceptor degeneration, comparable to the degenerative effect seen in mouse retinal explants [163]. This confirms the potential of retinal organoids as model system to recapitulate the side effects seen in humans, and further indicates that the positive effect of drugs against retinal disorders can be studied in this system. For example, the protective effect of ophthalmic supplements like vitamin E treatment has been confirmed in several studies in this context [163,187].

Apart from the utilization of whole organoids, RPE derived from PSCs can also be used as valuable target for drug-screening approaches, for example concerning the treatment of AMD [194](reviewed in [195]). A protective effect of the substance curcumin against oxidative stress on iPSC-derived RPE has been shown, in RPE from both AMD patients and healthy subjects [194]. Further, in gyrate atrophy—a retinal disorder affecting the RPE—iPSC-derived cells carrying a specific mutation have been used to demonstrate a responsiveness of the cells to vitamin B6 treatment [129]. In this case, dose titration experiments using the iPSC-RPE cell model were applied to find the optimal concentration of the drug, in order to avoid excessive treatment, which can otherwise lead to neurological side-effects. This is an example of a study providing the patients with the analyzed mutation an individualized therapy and therefore, a direct benefit [129].

The mentioned studies are only an excerpt of examples how pharmacological testing can make use of stem cell-derived retinal model systems. Especially, the utilization of whole retinal organoids and comparison of drug effects to effects seen in existing model systems will give important insights into the significance of *in vitro* retinal models for future drug screening.

5.4. Translational application

Current translational interests in new therapeutic approaches have set a focus on degenerative processes. Here, stem cell-based, regenerative therapies seem to become a fundamental part of it(reviewed in [196]). At first view, it seems consequential that recruiting and activating endogenous stem cell populations in a noninvasive manner might have several advantages over transplantation studies that use engrafted tissue or cell subtypes derived from exogenous sources [197–199]. In general, approaches mobilizing a patient's own resident and tissue-specific stem cells are considered as promising in every sense [200]. First, the risk of immune rejection and tumor formation from undifferentiated cells is reduced. Second, and perhaps more importantly, endogenous tissue-residing stem cells are usually far more prone to differentiate into the physiologically required cell type and integrate into the functional tissue. Further, degenerative phenotypes at early disease stages, which would normally not require transplantation yet, could be targeted for regeneration. However, in the CNS, the retina also represents an ideal target for stem cell-based transplantational studies because it is an immune-privileged site and various surgical techniques are established and available. In recent years, clinical approaches to transplant stem cell-derived retinal or non-retinal cell types have come to the fore. On the side of non-retinal cells, several clinical and pre-clinical studies are continuously undertaken, using adult mesenchymal stem cells (MSCs), for example [201]. These MSCs, administered through intravitreal injection, are considered to act *via* revascularization, immunomodulation, neuroprotection, and/or promoting regeneration (reviewed in [201,202]). Retinal cell types such as RPE or photoreceptor progenitors from fetal tissue have been successfully replaced in degenerated retinae of patients [199,203], albeit with variable benefit. With respect to pluripotent stem cells, numerous different techniques have been applied [161,204]. Of note, the first clinical I/II phase transplanting human-ESC-derived RPE cells in suspension was started with a promising outcome in AMD patients [205] and was followed by several similar studies throughout the world. Recent clinical trials from Masayo Takahashi and colleagues [206], transplanting RPE cells from PSCs on artificial layers, were highly appreciated in the press for using autologous iPSCs in the retina for the first time. This RPE “sheet” is positioned above the patient's RPE cell layer to replace the degenerated cell type and uphold the PRC survival and function [206]. Future trials may additionally aim for the replacement of several retinal cell types and layers in combination. Although huge progress has been achieved in the field of stem cell-based therapies in the retina, there is still a long way to go before a sustainable, reproducible, and safe treatment of retinal disorders can be conducted in a broad range using stem cells of any kind.

5.5. Organ and retina-on-a-Chip systems

Microfluidic Organ on a chip- technology has proven to become a powerful and promising system for *ex vivo* studies. Here, studies using microfluidic devices are conducted in a great variety, with the common aim to investigate cells and cell compositions, taking *in vitro* cultures closer to the physiological condition and providing an ideal platform for drug screening and individualized medicine (reviewed in [207,208]). Chip systems comprise numerous advantages compared to other *in vitro* systems. Just to name some of them, microfluidic devices with their structural variability allow for a tight environmental control in a system providing various built-in readouts, input/output channels for supplementing and analyses of fluids or enhanced viability. Moreover, cell differentiation and function can be greatly improved under microfluidic conditions as the environment, extracellular matrix and the mechanical cues of an organ can be closely engineered [209].In recent publications, numerous organ-on-a-chip approaches clearly demonstrated sophisticated cell systems paving the way for a future reform of stem cell based *in vitro* experiments. The combination of human stem cells, both of adult and pluripotent origin, represents a unique human model for a broad applicability in basic and clinical research [210–214](reviewed in [215–217]).

In the field of retina research, several approaches have been followed to date utilizing the chip technology. Microfluidic devices have been developed over the last years to investigate retina explants

and retinal cell lines [218–220] or the blood-retinal barrier [221,222], however, few studies yet deal with undifferentiated retinal cells or stem cells in microfluidic devices. One elegant study performed by Su et al. established a microfluidic device with mouse retinal progenitor cells aiming to recapitulate the synaptic wiring and regeneration. They succeeded in generating a system that comprised retinal signaling and synaptic connection. Furthermore, they were able to demonstrate retinal synaptic regeneration in-chip upon administration of synaptic inhibitory and excitatory molecules [223]. In another publication by Mishra et al., the authors generated a chip convenient for investigating the migratory abilities of retinal precursor cells in a diffusion-based system [224]. Microfluidic devices in retina research are able to overcome major hurdles of both, current *in vitro* models and animal models. Investigations of human retinal cells, explants or organoids will in the future be able to recapitulate several aspects of the physiological retina, thereby helping to search for new drugs, answer developmental questions and cell biology, represent a drug screening and individualized therapy platform.

5.6. Future perspectives

Stem cell derived cell subtypes play a promising role in clinical implications in various surroundings. Currently, *e.g.* stem cell derived RPE sheets are already used in clinical trials to both evaluate safety of such transplantations and further to find a cure for blindness. In many aspects, stem cell research has entered a new era exploring the field of organoid formation *in vitro*. These highly complex cell structures comprising various cell types of a specific organ or niche now allow for the investigation of human biology and pathogenesis from a unique and novel perspective. On the one hand, organoids represent a very promising system for transplantation into the human organism. Here, several organs may, at least in part, replace or restore degenerated tissue. Transplantation of *e.g.* liver or pancreas organoids have shown to be able to take over the function in animal models and may in the near future arrive in the clinics. On the other hand, microfluidic technology in combination with human stem cell derived cells and organoids are considered as an advanced tool for solving current and upcoming hurdles in regards of drug discovery, drug testing or toxicity screening [225,226]. Chip-technology thereby is evolving in its complexity and applicability. Emerging from a system with single cell subtypes, cell lines and animal explants, current research requires more complex systems. Several approaches using defined conditions in microfluidic devices have proven their usability and reliability in drug testing (reviewed in [227]). Moreover, worldwide initiatives are demanding for improved tissue-chips to solve, at least in part the devastating situation in case of using animal models (*e.g.* NIH in the US or the BMBF in Germany). The value of these human "model systems" will most probably become obvious in the near future and may revolutionize pharmacological platforms and approaches.

6. Closing remarks

Stem cell-based *in vitro* models are attracting more and more attention in various fields, ranging from disease modeling, as source for transplantational approaches to high-throughput drug testing. In this review, we present a variety of possible sources for retinal cell models; so far, however, only 3D-layered organoids derived from ESCs and iPSCs were able to reproduce major aspects of the complex and functional retina. This makes retinal organoid models extremely suitable for the sophisticated tasks required in modern and especially personalized medicine.

Despite the amazing features and abilities of the current retinal organoid culture models, they still face many drawbacks that they need to be solved in order to become a truly physiological model. In particular, the long culture time, the still immature photoreceptor outer segment morphology, and the introduction of immune cells and vascularization will be the goals of the near future that need to be achieved.

One way of creating a more complex and reproducible model could be the implementation of retinal organoids into an organ on a chip (OoC) context. OoC could help to standardize the model systems, bring in missing aspects of extracellular matrix, and eventually make testing easier and more reproducible.

Nonetheless, *in vitro* retinal models are on their way to become more physiological and could be a feasible replacement or addition to the current animal or explant models, especially where these cannot fully reproduce the human physiology.

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