Chapter 2
Human Pluripotent Stem Cells as In Vitro Models for Retinal Development and Disease

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Abstract Human pluripotent stem cells (hPSCs) provide unprecedented access to the earliest stages of retinogenesis that remain inaccessible to investigation, thereby serving as powerful tools for studies of retinal development. Additionally, the ability to derive hPSCs from patient sources allows for the modeling of retinal degenerative diseases in vitro, with the potential to facilitate cell replacement strategies in advanced stages of disease. For these purposes, many studies over the past several years have directed the differentiation of hPSCs to generate retinal cells using stochastic methods of differentiation, yielding all major cell types of the retina. In particular, these studies have favored the derivation of RPE, photoreceptors, and more recently retinal ganglion cells for disease modeling, drug screening as well as cell replacement purposes. More recently, advances in retinal differentiation methods have led to the generation of three-dimensional retinal organoids that recapitulate key developmental and morphological features of the retina, including the stratified organization of retinal cells into a tissue-like structure. This review provides an overview of retinal differentiation from hPSCs and their potential use for studies of retinogenesis as well as diseases that affect the retina.

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2.1 Introduction

The human retina is a multilayered tissue composed of an intricate network of several types of retinal neurons that function in an integrated manner to convert the incoming light stimulus into an electrical impulse, which will be propagated to the brain to be converted into an image. Consequently, any disease or injury affecting retinal neurons disrupts this visual circuit, resulting in blindness. Hence, a thorough understanding of the development and functions of the human retina will facilitate the development of successful therapies for retinal degenerative diseases. However, studies of the human retina are especially challenging as retinogenesis occurs early in gestation and remains largely inaccessible to investigation [1]. In this regard, human pluripotent stem cells (hPSCs), including human embryonic stem cells [2] and human induced pluripotent stem cells [3–5], provide a unique in vitro model capable of recapitulating the growth and diversification of developing retinal neurons.

hPSCs are self-renewing cells analogous to the inner cell mass/blastocyst stage of human development, which possess the ability to generate all cell types of the body. Therefore, hPSCs can be used to study even the earliest events of retinogenesis and generate limitless numbers of retinal neurons for translational applications [6–15]. While advancements in hPSC-retinal differentiation protocols over the last decade have led to the successful generation of all types of retinal neurons [9, 16–27], these cells have traditionally been differentiated in a manner that lacked the ability to assemble into a multilayered retinal-like structure. This lack of cellular organization not only affects the ability to faithfully recapitulate the events of retinogenesis as an in vitro model, but may also impact the quality and functionality of retinal cells generated for future translational applications, including disease modeling and cell replacement.

More recently, a fundamental shift in retinal differentiation protocols has developed which allows for the organization of hPSC-derived retinal neurons into an organized, multi-layered retinal-like structure [21, 28–30]. These resultant populations, known as retinal organoids, are composed of retinal neurons arranged in a stratified manner that recapitulates the spatial and temporal patterning of native retinal tissue [21, 23, 28–39]. Thus, such hPSC-derived retinal organoids will likely serve as more effective in vitro models with which to recapitulate earliest events of retinogenesis. Furthermore, these retinal organoids may enhance the application of hPSCs for disease modeling and cell replacement. To serve in these capacities, however, refinements in the differentiation of retinal organoids will be needed, with improvements in these protocols likely to be inspired by our growing understanding of the regulatory factors at play in the developing retina in vivo.

2.2 Development and Organization of the Vertebrate Retina

The retina is a complex multilayered tissue that originates from the developing diencephalon and consists of six neuronal cell types that work in a coordinated fashion to perceive and interpret incoming visual information [40, 41]. Based on the orientation of retinal cells, the retina can be broadly classified into three layers: (1) the outer nuclear layer consisting of the photoreceptor cells, including the rods and cones, (2) the inner nuclear layer consisting of the interneurons, namely bipolar cells, amacrine cells, and horizontal cells, and (3) the ganglion cell layer consisting of the retinal ganglion cells whose axons extend to form the optic nerve [42, 43]. In addition to these neuronal cell types, Muller glia are the primary glial cells of the retina, with cell bodies in the inner nuclear layer and processes traversing the length of the retina, providing necessary architectural and functional support. Additionally, photoreceptors are supported and nourished by the retinal pigmented epithelium (RPE), a sheet-like layer of epithelium located below the photoreceptor layer. Retinal neurons are intricately connected through a network of synapses, with connections between the photoreceptors, bipolar cells, and horizontal cells, referred to as the outer plexiform layer. Similarly, the inner plexiform layer represents the dense fibrils between the ganglion cells, bipolar cells, and amacrine cells.

This structure forms a highly regulated pathway for visual transduction, which is critical to the functioning of the retina [44]. Briefly, incoming light is focused onto the retina via the cornea and lens, where it first interacts with the photoreceptors in the outermost layers of the retinal tissue. These photoreceptors convert the visual light into an electrical stimulus via the phototransduction pathway, which is then transmitted to the retinal ganglion cells via the interneurons of the retina. Finally, the ganglion cells extend their long axons via the optic nerve and synapse with their postsynaptic targets, including the superior colliculus and the lateral geniculate nucleus. Relays to cortical areas responsible for signal integration enable vision. Overall, the function of the retina depends on all its components working in a sequential manner to integrate and transmit the visual information to the brain. Consequently, any disruption in this visual circuit due to injury or disease results in loss of vision or blindness. As such, the use of hPSCs provides a powerful tool with which to study the development of the retina, as well as disruptions to retinal function resulting in vision loss [45]. However, modeling the functions of the retina and its pathophysiology requires the differentiation and organization of these cells in a manner which closely recapitulates the native retina, necessitating a thorough understanding of mechanisms associated with retinal development in vivo.

Retinal development is determined by the combinatorial actions of growth factors as well as transcription factors, which not only specify retinal cell types but also determine their spatio-temporal location. Retinogenesis begins early in gestation and the first morphological evidence of the retina is seen during neurulation [46]. As the developing neural plate forms the neural tube, optic grooves emerge on either side of the diencephalon. These grooves, now known as optic vesicles, evaginate toward the surface ectoderm, resulting in reciprocal signaling between these struc-

tures. This reciprocal exchange of signals leads to the induction of the retina from the distal optic vesicle and the formation of the lens placode from the ectoderm. Consequently, the proximal optic vesicle is induced by the surrounding mesenchyme to form the retinal pigmented epithelium (RPE). Following specification of the optic vesicle, these cells acquire a multipotent progenitor identity and will subsequently multiply and differentiate into all cell types of the retina (Fig. 2.1). Retinal cell genesis is specified in an evolutionally-conserved order, which is dictated by the competence of retinal progenitors, and a combination of exogenous signaling gradients as well as endogenous transcriptional regulation [47–49]. Based on this model, studies of retinal development in model systems have demonstrated that ganglion cells, horizontal cells, and cone photoreceptors are the earliest-born retinal cell types. Amacrine cells are specified slightly later in development, followed by rod photoreceptors, while bipolar and muller glia cells are the last cell types to develop in the retina. Retinal development and maturation continues throughout gestation and visual synapses continue to mature after birth. Overall, the specification of the retina from its early diencephalic origins follows a tightly conserved order of events.

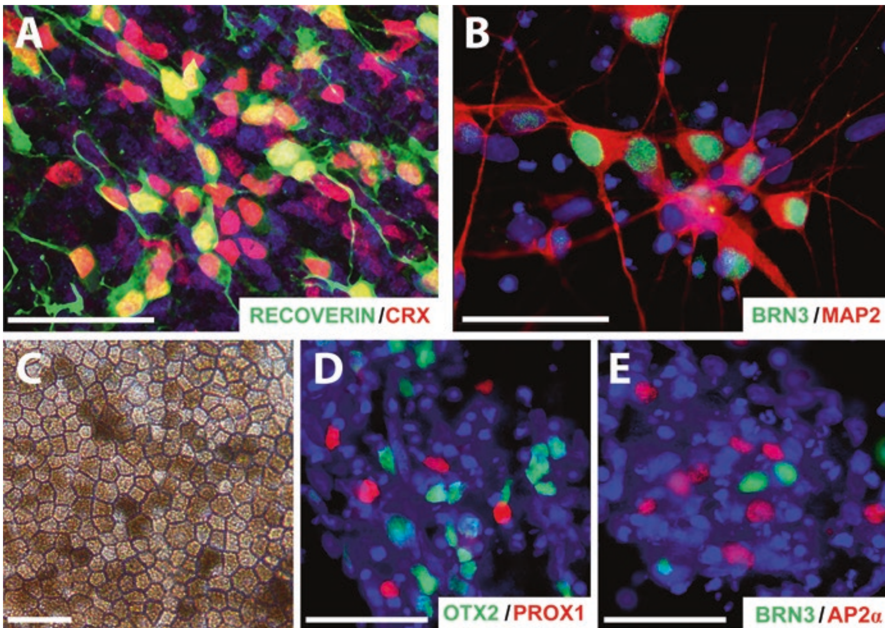

Fig. 2.1 hPSCs generate retinal cells using stochastic methods of differentiation. hPSCs were directed to a retinal fate using a stepwise protocol, where retinal neurons were specified in a conserved, temporal sequence. Within 70 days of differentiation, photoreceptors were readily identified by the expression of photoreceptor-specific markers CRX/RECOVERIN (**a**), while ganglion cells expressed BRN3 and extended MAP2 positive neurites (**b**). hPSCs-derived RPE demonstrate characteristic pigmentation and hexagonal morphology, seen via bright field microscopy (**c**). Additionally, hPSC-derived interneurons such as horizontal cells and amacrine cells could be identified via PROX1 and AP2α expression respectively. Scale bars equal 40 μm

Likewise, to properly and reliably direct the differentiation of hPSCs to a retinal fate, knowledge and application of these developmental events to cellular differentiation protocols is essential.

2.3 In Vitro Studies of Retinal Development Using hPSCs

The development of the human retina is initiated at some of the earliest stages of gestation, making the study of these critical cell fate determination events difficult. Given their pluripotent nature, hPSCs may provide a unique and novel tool for the study of these early developmental events by serving as comprehensive models of the major stages of human retinogenesis, even at stages that would be otherwise inaccessible to investigation in the embryo. With the resultant retinal cells, the potential then exists for their translational application, including cell replacement approaches as well as the ability to model and study retinal degenerative diseases in a dish when derived from specific patient sources. In order to serve in this capacity, however, these cells must be directed to differentiate toward a retinal lineage in a step-wise process that faithfully recapitulates the major stages of retinogenesis in vivo [50]. As such, numerous efforts have been made over the last decade focused on the derivation of retinal cells from hPSCs, often adopting critical principles of developmental biology to guide the differentiation process (Table 2.1). Initial work in this field focused upon the differentiation of retinal cells by inhibiting BMP and WNT signaling in the presence of IGF-1 [9, 19, 68]. Similarly, other groups have been successful in achieving retinal differentiation through the inhibition of WNT and Nodal signaling [17, 18, 24, 25, 69]. Subsequently, efforts relying upon the default adoption of a rostral neural fate in the absence of specific morphogenic factors led to the development of discrete retinal progenitor cell populations, which would later give rise to retinal neurons in a temporally appropriate manner [21–23, 26, 27, 63].

While these protocols provided the ability to generate all the major cell types of the retina, most of the early focus has favored the generation of RPE and photoreceptor cells as many retinal diseases primarily affect these outer retinal cells, resulting in their degeneration and subsequent loss of vision. Additionally, RPE and photoreceptors possess unique phenotypic markers and functional properties that enables their ease of identification in vitro, which is often lacking for other cell types of the retina. Among the earliest retinal differentiation studies, RPE was first observed to be spontaneously differentiated from hPSCs in relatively high numbers [51]. These cells were initially identifiable in cultures of differentiating stem cells due to the accumulation of melanin pigment within these cells that could be readily visualized. Further confirmation of RPE differentiation was provided by their characteristic hexagonal shape and upon isolation of these cells, they commonly expressed a full complement of RPE-associated features [21, 52, 54–56, 59, 60, 70–77]. Similarly, photoreceptors were among the first retinal neurons to be

Table 2.1 Summary of stochastic methods of retinal differentiation from hPSCs

Authors	Cell types observed	Signaling factors	Functional characteristics
Klimanskaya et al. [51]	RPE	bFGF LIF KOSR	
Lamba et al. [19]	Amacrine cells Bipolar cells Horizontal cells Photoreceptors Retinal ganglion cells	Dkk1 IGF1 Noggin	Calcium response to glutamate and NMDA
Banin et al. [16]	Photoreceptors	bFGF EGF Noggin	
Osakada et al. [24]	Amacrine cells Bipolar cells Horizontal cells Immature muller cells Photoreceptors RPE Retinal ganglion cells	Activin-A aFGF bFGF DAPT Dkk1 FBS Lefty Retinoic acid Shh Taurine	
Carr et al. [7, 52]	RPE	bFGF Hydrocortisone KOSR Taurine T3	Photoreceptor outer segment phagocytosis
Meyer et al. [22]	Photoreceptors RPE	Default neural specification	
Idelson et al. [53]	RPE	KOSR Activin-A TGFβ1 SB431542 bFGF	
Buchholz et al. [54]	RPE	bFGF KOSR FBS	Photoreceptor outer segment phagocytosis
Mellough et al. [20]	Photoreceptors RPE	Activin A bFGF Dkk1 IGF1 Lefty Noggin Retinoic acid Shh T3 Taurine	

(continued)

Table 2.1 (continued)

Authors	Cell types observed	Signaling factors	Functional characteristics
Singh et al. [55, 56]	RPE	bFGF EGF FBS FGF2	Photoreceptor outer segment phagocytosis Polarized secretion of growth factors Calcium responses to ATP ATP, TER measurements
Tucker et al. [57]	Photoreceptors RPE	aFGF bFGF DAPT Dkk1 IGF1 Lefty Noggin Retinoic acid Shh T3 Taurine	
Riazifar et al. [58]	Retinal ganglion cells	bFGF DAPT FBS KOSR	Inward/outward currents Action potential response to CNQX
Ferrer et al. [59]	RPE	Activin A Hydrocortisone KOSR Nicotinamide Noggin SB431542 Taurine Triiodothyronine	Intracellular calcium responses Respond to changes in potassium and ATP concentrations
Maruotti et al. [60]	RPE	Chetomin Nicotinamide	
Zhou et al. [61]	Photoreceptors	COCO Dkk1 FGF2 IGF1 Noggin T3	
Sluch et al. [62]	Retinal ganglion cells	FGF-A FBS Forskolin Taurine	Firing of action potentials Response to AMPA/NBQX Mitochondrial movement through RGC axons
Ohlemacher et al. [63]	Retinal ganglion cells	Default neural specification	Firing of action potentials Hyperpolarized resting membrane potential Inward/outward ionic currents

(continued)

Table 2.1 (continued)

Authors	Cell types observed	Signaling factors	Functional characteristics
Barnea-Cramer et al. [64]	Photoreceptors	BDNF CNTF DAPT Insulin Noggin Retinoic acid	
Gill et al. [65]	Retinal ganglion cells	bFGF Dkk1 IGF1 KOSR Noggin	Firing of action potentials Inward/outward currents Axonal transport of mitochondria
Teotia et al. (2017)	Retinal ganglion cells	BDNF cAMP CNTF Cyclopamine DAPT FGF8 Follistatin Forskolin NT4 Shh Y27632	Inward/outward currents Fire action potentials
Sluch et al. [66]	Retinal ganglion cells	DAPT Dorsomorphin Forskolin Glutamax IDE2 LDN-193189 Nicotinamide Noggin SB431542	Efficient immunopurification of RGCs In vitro axonal injury model
Langer et al. [67]	Retinal ganglion cells	Default neural specification	Identification of RGC subtypes Use of single cell RNAseq to elucidate RGC subtypes and novel subtype markers

identified due to the large number of photoreceptor-specific markers that have been previously identified in retinal development studies [9, 19, 20, 22, 24, 25, 78].

More recently, some efforts have focused upon the differentiation of retinal ganglion cells from hPSCs. These cells have been somewhat more difficult to definitively identify in differentiating cultures as they lack any truly specific markers to separate them from some other neuronal populations. However, the ability to identify these cells has been facilitated in recent years by following their differentiation through a retinal progenitor intermediary or via the use of fluorescent reporters [58, 62, 63, 65, 79–82]. Additionally, some studies have demonstrated the ability to derive all the major neuronal cell types of the retina, including interneurons, although these cells have not been extensively characterized to date [21, 22, 25–27, 29, 61, 63, 68, 69, 83].

While the above methodologies have been highly successful for the derivation of all of the major types of retinal neurons, this differentiation often occurred as a somewhat heterogenous population of retinal cells. This differentiation allows for the ability to study many features within individual cells, but does not account for the critical interactions between neurons of the retina which are necessary for their proper maturation and function. Furthermore, many disorders of the retina result from the loss of connectivity between cells, making the study of these disorders more difficult in heterogeneously arranged cultures. To overcome these shortcomings, efforts have been directed toward the differentiation of retinal cells from hPSCs in a manner which closely mimics the development and three-dimensional organization of the retina. Initially, studies described the ability of hPSCs to differentiate toward a retinal lineage in a step-wise fashion, yielding three-dimensional structures closely resembling the optic vesicle and optic cup stages of retinogenesis [21–23, 29]. Subsequently, further efforts expanded upon these early results to generate three-dimensional structures termed retinal organoids that were found to effectively recapitulate the spatial and temporal organization of the various neuronal cell types of the retina, resulting in a stratified, multilayered structure [28, 30, 34–38].

2.4 Applications of Retinal Organoids for Modeling Human Development

With the goal of effectively recapitulating the complex organization and interplay between the different types of neurons of the retina, studies within the past few years have described the ability of hPSCs to differentiate toward a retinal lineage in a step-wise fashion [17, 22, 68]. The resultant populations of cells have yielded structures that closely resemble the developing optic cup, with enriched populations of retinal progenitor cells discretely arranged into a cup-like structure (Fig. 2.2). Subsequent efforts have expanded upon these early results to generate retinal organoids that effectively recapitulate the spatial and temporal organization of the various neuronal cell types of the retina (Table 2.2). As a result, these retinal organoids provide a powerful and novel tool for studies of the earliest stages of human retinal development.

As compared to early methods of retinal differentiation from pluripotent cells, retinal organoids offer several advantages as an in vitro model of retinogenesis. Importantly, these organoids can self-assemble into discrete three-dimensional structures with major classes of retinal neurons arranged into distinct layers similar to their organization within the retina [21, 28–31, 33, 35–39]. The differentiation of these retinal organoids progresses through all the major stages of retinogenesis, including stages analogous to the eye field, optic vesicle, and optic cup, thereby allowing for the ability to visualize some of the earliest events of human retinal development. Similar to embryonic retinogenesis [50], differentiation of resultant cells within retinal organoids has been demonstrated to follow a conserved sequence of events, with early-born cell types such as RGCs among the first retinal neurons

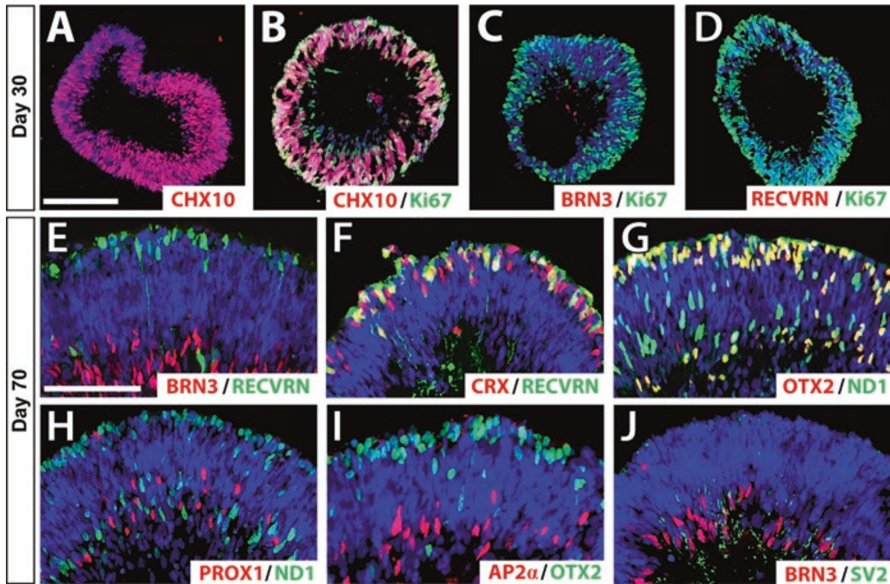

Fig. 2.2 hPSCs can be directed to generate retinal organoids using three-dimensional differentiation approaches. hPSCs were directed to generate retinal organoids in a stepwise manner analogous to major stages of retinogenesis. Optic vesicle-like retinal organoids expressed retinal progenitor markers, CHX10, PAX6, and cell proliferation marker Ki67 after 1 month of differentiation (**a**–**d**) while photoreceptor marker RECOVERIN and ganglion cells marker BRN3 were seldom seen at this early stage of development. After 2 months of differentiation, retinal organoids acquired a cup-like appearance and retinal cells were arranged in a stratified manner. Photoreceptor markers (CRX, RECOVERIN, OTX2, and ND1) occupied apical layers of the organoids (**e**–**i**), horizontal and amacrine cells (PROX1, AP2α) in the middle and ganglion cells (BRN3, SV2) occupied basal layers within the organoids (**e**–**j**). Scale bars equal 150 μm for **a**–**d** and 100 μm for **e**–**j**

to be specified, while later-born cell types such as rod photoreceptors among the last [21, 23, 28–31, 33, 34, 37, 85].

Retinal cells occupy strategic positions within the adult retina, with ganglion cells residing in the innermost layers of the retina, whereas photoreceptor cells closely associate with RPE and form the outermost layers. The spatial arrangement of retinal neurons and their synaptic connections linking them together are critical to their proper function and as such, retinal cells derived from hPSCs should similarly recapitulate this level of organization. While traditional methods of differentiation have allowed for the successful generation of all the major cell types of the retina, these approaches have lacked the ability of retinal cells to assemble into a layered structure. These shortcomings of traditional approaches have been overcome by the development of retinal organoids, which allow for the maintenance of cell–cell contacts between retinal neurons [21, 23, 28–30, 32, 33, 35, 37, 38, 85]. These organoids formed a pseudostratified epithelium-like structure which allows the retinal cells to

Table 2.2 Summary of retinal organoid differentiation from hPSCs

Authors	Cell type-specific markers expressed				Signaling factors	Structural characteristics	Functional characteristics
	Photoreceptors	Ganglion cells	Amacrine/ horizontal cells	Bipolar cells			
Meyer et al. [21]	CRX Recoverin	BRN3	Calretinin	VSX2 PKCα	Default neural specification	Establishment of optic vesicle-like organoids	Photoreceptors responsive to 8-Br-cGMP
Philips et al. [29]	CRX Recoverin Rhodopsin S-opsin	BRN3 HuC/D TUJ1	Calretinin	PKCα	Default neural specification	Expression of synaptic and gap junction proteins	
Nakano et al. [28]	CRX NRL Recoverin Rxr-gamma	BRN3 PAX6 TUJ1	Calretinin Ptf1a	PKCα	CHIR99021 DAPT IWR1 Retinoic acid SAG Y-27632	Self-organization of hPSCs into optic cup like structures Multilayered retina with roads and cones	
Zhong et al. [30]	CNG α-and β $G_{T1\alpha}$ Guanylate cyclase 1 OTX2 PDE6-α Recoverin Rhodopsin S-opsin	BRN3 HuC/D	AP2α HuC/D PROX1	VSX2	FBS Taurine Retinoic acid	Outer segment disc formation Outer limiting membrane Inner segments Mitochondria and basal bodies in photoreceptors	Photoreceptors exhibited rudimentary responses to light

(continued)

Table 2.2 (continued)

Authors	Cell type-specific markers expressed				Signaling factors	Structural characteristics	Functional characteristics
	Photoreceptors	Ganglion cells	Amacrine/ horizontal cells	Bipolar cells			
Reichman et al. (2017)	CD73 Cone arrestin CRX L/M-Opsin OTX2 Recoverin Rhodopsin	BRN3A	AP2α Calretinin LIM1	Glutamine Synthase PKCα SOX9	DAPT FGF2	Laminar organization of retinal cells	
Mellough et al. [34]	Basoon Calbindin28 CRX L/M-Opsin Recoverin Rhodopsin S-Opsin	CALB1 HuC/D Islet1 TUJ1	CALB1 HuC/D TUJ1	CALB1	IGF-1	Cilia and melanosomes in RPE, nascent outer segment discs, basal body complex in photoreceptors.	Photoreceptors responsive to 8-Br-cGMP
Ohlemacher et al. [23]	CRX OTX2 Recoverin	BRN3 MAP2			Default neural specification	Morphological isolation of retinal organoids apart from forebrain populations	
Singh et al. [36, 76]	CRX Recoverin	BRN3B	Calretinin LGR5 NF200		bFGF Dkk1 IGF-1 FGF9 Noggin	Laminar organization of retinal cells Migration of axons within retinal tissue	Electrophysiological responses in inner retinal neurons

Kaewkhaw et al. [31]	BLIMP1 CRX NRL S-Opsin Recoverin	BRN3B CALB1	CALB1 CALB2	CALB1 PKCα	Matrigel FBS Retinoic acid SAG	Laminar organization of retinal cells	
Völkner et al. [37]	CRX OTX2 Recoverin Rhodopsin SALL3	BRN3 HuC/D Islet1 TUJ1	BHLHB5 CALB1 CALB2 EBF3 HuC/D PROX1 TFAP2A TUJ1	PRKCA SOX9 VSX2	DAPT	Laminar organization of retinal cells	
Lowe et al. [33]	L/M-Opsin OTX2 Recoverin Rhodopsin	CALB1 ISLET1/2 TUJ1	CALB1 TUJ1	CALB1 VSX2	Y-27632	Cilia, basal bodies, mitochondria and membrane discs in photoreceptors	
Parfitt et al. [84]	Arrestin L/M-Opsin Recoverin Rhodopsin S-opsin	BRN3 HuC/D	HuC/D		CHIR99021 IWR1 Retinoic acid SAG Y-27632	Cilia and mitochondria in inner segments	

(continued)

Table 2.2 (continued)

Authors	Cell type-specific markers expressed				Signaling factors	Structural characteristics	Functional characteristics
	Photoreceptors	Ganglion cells	Amacrine/ horizontal cells	Bipolar cells			
Wiley et al. [39]	CRX L/M-Opsin NRL Recoverin Rhodopsin S-Opsin	HuC/D	HuC/D	PKCα	CHIR99021 DAPT ECM IWR1 SAG	Laminar organization of retinal cells	
Wahlin et al. [38]	CRX L/M-Opsin OTX2 Recoverin Rhodopsin Opsin rod RET-P1 S-Opsin	BRN3 NF200			DAPT IWR1 Retinoic acid SAG	Microvilli on RPE Photoreceptor inner segment with cilia, basal body, mitochondria Rudimentary outer segment with discs Photoreceptor outer limiting membrane Synaptic ribbons	Excitatory and inhibitory neurotrasmitter responses HCN-type current in cones Voltage-dependent release of synaptic vesicles
Gonzalez-Cordero et al. (2017)	ABCA4 Arrestin CRX L/M Opsin NRL PRPH2 Onecut-1 OTX2 Recoverin Rhodopsin RETGC S opsin				FBS Glutamax Glutamine Retinoic Acid Taurine	Inner segments and connecting cilia Nascent outer segments and presynaptic structures Transplantable cone photoreceptors	

mature in both a temporal and spatial fashion, with ganglion cells specified in basal laminae of the organoids while photoreceptors occupy apical regions.

The three-dimensional nature of organoids also likely aids in the functional maturation of retinal neurons, which has been largely limited in retinal cells derived using traditional differentiation methods. While these retinal cells differentiated by traditional approaches commonly express a variety of features associated with all of the major cell types of the retina, these cells lacked the structural and functional differentiation typically associated with more mature retinal neurons. The use of three-dimensional retinal organoids allows for the acquisition of more advanced features of differentiation within these cells including enhanced outer segments and the ability to respond to light stimuli, presumably due to their ability to interact and self-organize with neighboring cells. Further refinements of these organoid cultures have also involved the addition of external signaling molecules in long-term cultures to further guide their differentiation [28, 30, 32]. This has been particularly true for photoreceptors, which have been the most extensively studied cell type derived within retinal organoids. The experimental manipulation of critical signaling pathways within retinal organoids has led to refinements in photoreceptor differentiation, including accelerated differentiation as well as increased expression of phototransduction proteins. Photoreceptors derived in this fashion exhibited characteristic bulb-like structures at their tips, demonstrated membranous disc-like structures in regions resembling outer segments, and occasionally displayed electrophysiologic responses to light stimuli [30, 38].

2.5 Application of hPSC-Derived Cells for Retinal Disease Modeling

Beyond the applications of hPSCs for modeling retinal development, these cells also serve as powerful and unique platforms for the study of human retinal degenerative diseases. Due to the degeneration of specific populations of retinal neurons, these diseases are characterized by loss of vision and eventual blindness. Retinal degenerative diseases can be most readily classified into diseases that affect cells of the outer retina or those affecting the inner retina, most notably age-related macular degeneration and glaucoma, respectively [86, 87]. Traditionally, the ability to study the progression of these disease states has been limited to animal models. While these animal models have led to significant advances in our understanding of retinal disease progression [88–94], important differences exist between the retinas of animal models and humans, including the prevalence of rods and cones as well as the presence of a macula in humans. Furthermore, studies in humans have been largely limited to postmortem retinal tissue or to retinal imaging approaches that lack the resolution to examine individual cells. While these studies have been informative about the end-result of disease pathology, the approach necessarily limits the ability to better understand disease progression within individual cells.

In order to overcome these shortcomings for studies of retinal degenerative diseases, recent research has focused on the use of hPSCs to model and understand disease progression (Table 2.3). When generated from patients with a known genetic basis for retinal degeneration, hPSCs provide an infinite supply of cells for the derivation of the affected cell type, and can thereby serve as powerful tools to study the disease phenotype [85, 110, 111]. Over the last several years, studies have utilized hPSCs for studies of degenerative diseases of the retina, with a particular focus on those diseases that affect RPE and photoreceptors [11, 15, 21, 56, 60, 70–72, 95, 96, 98, 101, 105, 109, 112–119]. These cells are often affected in retinal degenerative diseases such as age-related macular degeneration, and the derivation of these cells has been extensively characterized through hPSC retinal differentiation protocols. Such approaches have helped to demonstrate the improper function and/or reduced survival of RPE and photoreceptors in patient-derived cells, thereby providing insight into potential mechanisms underlying the loss of these retinal cell types [11, 21, 53, 56, 101, 105, 115, 116]. Furthermore, patient-derived hPSCs have also been utilized to identify novel genetic variants underlying retinal degeneration, highlighting the potential to target this area for the development of therapies [96, 98].

While diseases affecting cells of the outer retina have been extensively studied with hPSCs, studies related to diseases affecting inner retinal neurons have been largely limited. Of the diseases affecting inner retinal neurons, the most common is glaucoma with a current incidence of greater than 60 million people worldwide [120, 121]. Glaucoma results in the degeneration of retinal ganglion cells (RGCs), leading to a decreased connectivity between the eye and the brain and subsequent

Table 2.3 Selected demonstrations of retinal disease modeling with hPSCs

Authors	Disease modeled	Cell types studied	Disease phenotype	Therapeutic approach	
				Gene correction	Drug screening
Jin et al. [95]	Retinitis Pigmentosa	Photoreceptors	ER stress Oxidative stress		✓
Meyer et al. [21]	Gyrate Atrophy	RPE	Enzymatic defect	✓	✓
Tucker et al. [96]	Retinitis Pigmentosa	Photoreceptors	Alu mutation in MAK gene		
Singh et al. [55, 56]	Best disease	RPE	Phagocytosis defects Oxidative stress Altered calcium homeostasis		
Minegishi et al. [97]	Glaucoma	Neural cells	Increased protein deposits and insolubility		✓
Lustremant et al. [98]	LCA	RPE	Gene polymorphism Oxidative stress		
Tucker et al. [99]	Glaucoma	Retinal ganglion cells	Autophagy defects		
Yang et al. [100]	AMD	RPE	Oxidative stress		

(continued)

Table 2.3 (continued)

Authors	Disease modeled	Cell types studied	Disease phenotype	Therapeutic approach	
				Gene correction	Drug screening
Cereso et al. [101]	Choroideremia	RPE	Biochemical defect Under prenylation of protein	✓	
Yoshida et al. [102]	Retinitis pigmentosa	Photoreceptors	Increased ER stress Increased apoptosis Autophagy defects	✓	✓
Burnight et al. [6]	LCA	Photoreceptors	Fewer and shorter cilia	✓	
Li et al. [103]	Retinitis pigmentosa	RPE	Disorganized RPE Loss of apical microvilli Reduced pigmentation	✓	
Lukovic et al. [11]	Retinitis pigmentosa	RPE	Phagocytosis defects		
Schwarz et al. [104]	Retinitis pigmentosa	RPE	Trafficking defects in cilia Loss of protein Disrupted golgi cohesion	✓	
Singh et al. [36, 76]	Best disease	RPE	Increased oxidation Phagocytosis defects		✓
Moshfegh et al. [105]	Best disease	RPE	Chloride channel defect		
Chen at al. [106]	Dominant optic atrophy	RGCs	Increased apoptosis		✓
Ohlemacher et al. [63]	Glaucoma	Retinal ganglion cells	Increased apoptosis		✓
Parfitt et al. [84]	LCA	RPE Photoreceptors	Cilia defects Abnormal protein splicing	✓	
Saini et al. [107]	AMD	RPE	Upregulated complement and inflammatory markers		✓
Ramsden et al. [108]	Retinitis pigmentosa	RPE	Phagocytosis defects	✓	✓
Teotia et al. [82]	Glaucoma	Retinal ganglion cells	Shortened neurites, immature activity, reduced expression of guidance cues		
Hallam et al. [109]	AMD	RPE	Increased inflammation and cell stress, accumulation of liquid droplets, impaired autophagy		

loss of vision. The ability to derive RGCs from hPSCs has been a more recent area of investigation, which now allows for the application of these cells for studies of retinal degenerative diseases affecting the inner retina [58, 62, 63, 81, 82, 97, 99, 122]. Recently, efforts have focused on the use of hPSCs from patients with genetic determinants of degenerative diseases that directly affect the RGCs, such as gene mutations underlying some forms of normal tension glaucoma and dominant optic atrophy. Interestingly, upon the differentiation of these cells, RGCs from patient sources exhibited increased apoptosis, thereby allowing for subsequent studies of disease mechanisms leading to degeneration of RGCs [63, 106].

While traditional retinal differentiation protocols have been highly successful in modeling certain features of some retinal degenerative diseases, the resultant retinal cells differentiate in a manner that lacks any three-dimensional organization that mimics how cells are arranged into retinal tissue. Retinal organoids may serve as an improved model for studies of retinal disease modeling, allowing for the interaction between different cell types and therefore providing the ability to assess the effects of degeneration on the entire tissue. While such an ability has yet to be demonstrated for retinal organoids, the use of cerebral organoids for disease modeling has provided an important proof of principle and have been particularly successful for some of the effects of cerebral diseases, such as microcephaly and lissencephaly [123–125]. In the near future, it is likely that retinal organoids will be applied for the study of retinal degenerative diseases. As recent studies have demonstrated the successful organization and maturation of photoreceptors within retinal organoids [21, 29, 30, 34, 38, 76], disease-modeling approaches will most likely be applied for outer retinal diseases. Recent studies utilizing hPSC-derived retinal organoids have primarily utilized a genetic basis versus an idiopathic basis for retinal degenerative diseases [39, 76, 84]. Additionally, further improvements to retinal organoids will likely be necessary to be able to apply them to a wide variety of retinal degenerative diseases. For example, hPSC-derived retinal organoids do not demonstrate a macula-like region or a functioning RPE layer, they are currently suited to model diseases that affect peripheral photoreceptors [39, 76, 84]. Further improvements in the differentiation methods to also include the characterization and maturation of inner retinal neurons will enable the study of diseases to affect ganglion cells with retinal organoids.

2.6 Drug Screening with hPSC-Derived Retinal Cells

When derived from individual patient populations, particularly those with a known genetic basis underlying retinal disease, hPSCs possess the ability to recreate certain features of the disease phenotype and model the degeneration associated with retinal diseases. With the resulting data accumulated from such studies, these cells can then be utilized for the development of therapeutic approaches for retinal degenerative diseases [111, 119, 126–129]. Following the directed differentiation of patient-derived hPSCs to a retinal fate, drug screening efforts can be targeted to an

affected retinal cell type, providing a platform for assessing the ability of candidate compounds to rescue the disease phenotype.

The use of patient-derived hPSCs for drug screening has been particularly successful for degenerative diseases that affect the outer retina, whose cells have been routinely derived and extensively characterized from hPSCs [6, 11, 21, 76, 84, 102–104, 107, 108, 116, 117, 130]. Photoreceptors and RPE are the most common cell types affected in many retinal degenerative diseases such as age-related macular degeneration (AMD), where the loss of photoreceptors combined with dysfunctions in RPE leads to loss of vision. As patient-derived RPE has been shown to recapitulate some of the hallmark features of AMD, including elevated expression of inflammatory factors and defective oxidative stress responses, recent studies have utilized hPSC-derived RPE as a platform for the screening of candidate drugs to assess the ability to improve their survival [56, 59, 76, 100, 131]. The results of these studies have enabled the identification of select compounds as potential neuroprotective agents that can alleviate RPE degeneration [107]. Similarly, hPSC-derived retinal cells have also been utilized for drug screening purposes as a means to alleviate photoreceptor loss due to retinitis pigmentosa, with results indicating that hPSC-derived photoreceptors were able to recapitulate the disease phenotype and upregulate markers of oxidative stress, lipid oxidation, and apoptosis [11, 57, 102, 103, 108]. Treatment of the degenerating rod photoreceptors with antioxidant vitamins effectively increased photoreceptor survival.

While hPSC differentiation strategies initially emphasized the cells of the outer retina, recent refinements in differentiation protocols have enabled the stepwise differentiation and identification of inner retinal neurons, particularly RGCs [58, 62, 63, 79, 81, 82, 106]. RGCs serve as the critical connection between the eye and the brain to transmit visual information, and their degeneration is part of a spectrum of diseases known as optic neuropathies, resulting in vision loss and eventual blindness. RGCs differentiated from hPSCs, particularly when derived from patient-specific sources, allow for the ability to screen new drug compounds and develop personalized treatment profiles for optic neuropathies [63, 106]. As a proof of principle, recent studies have successfully demonstrated the ability to faithfully recapitulate some of the degenerative processes associated with optic neuropathies in hPSC-derived RGCs, with subsequent drug screening approaches enabling the identification of neurotrophic factors capable of rescuing RGC degeneration [63].

While a number of studies have successfully demonstrated the ability to screen compounds for their neuroprotective effects on hPSC-derived retinal cells, these approaches have focused on isolated cells lacking any three-dimensional organization reminiscent of retinal tissue. With the advent of retinal organoids, hPSCs can be directed to differentiate in a manner that recapitulates the architecture, spatial connectivity and functioning of the retina, and may therefore be better suited for drug screening purposes. Given the more detailed demonstration to date of photoreceptor differentiation and organization in the outer layers of retinal organoids, these cells are likely better suited for drug screening applications for photoreceptor diseases. In contrast to outer retinal diseases, retinal organoids can also be used to test and

develop therapies for inner retinal neurons such as RGCs, which are primarily affected in optic neuropathies.

2.7 hPSC-Derived Retinal Cells as a Vehicle for Cell Replacement

While early stages of retinal degenerative diseases may be effectively studied with hPSCs, and subsequently drug screening approaches may aid in the neuroprotection of these degenerating cells, the irreversible loss of retinal neurons in later stages renders such measures ineffective, resulting in severe vision loss and blindness. In such cases, attempts to replace degenerated cells through transplants of healthy retinal cells constitute the only remaining effective option to restore some visual function [129, 132]. The transplantation of cells into the retina represents a more feasible option for cell replacement when compared to other cells of the nervous system, as the relative ease of accessibility of the retina and its reduced immunological response will likely facilitate cell replacement [133, 134]. To aid in this goal, hPSCs can serve as a renewable source of stem cells for the differentiation of retinal cells for a variety of translational approaches to retinal repair. Transplants of hPSC-derived retinal cells can assist in neuroprotection, particularly at earlier stages of the disease process, and can lead to potential delay in disease progression. At later stages of the degenerative process, hPSC-derived retinal cells can serve as a source for repopulation of the retina following the loss of host neurons.

Several studies have examined the use of hPSC-derived photoreceptors for cell replacement in diseases that affect the outer retina, with the goal to replace the degenerating neurons with their functional equivalents [9, 39, 64, 135, 136]. Initial studies focused on transplantation of undifferentiated retinal stem cells into animal models, which could integrate into many layers of the retina and exhibit neuronal morphologies [137–139]. However, these cells were often limited in number and their ability to be expanded, and rarely exhibited any ability to give rise to photoreceptor cells. As an alternative, more recent efforts have focused upon the ability of hPSC-derived photoreceptor cells for cell replacement. Upon transplantation, several groups have demonstrated the ability of these cells to integrate into the host retina and form connections with other retinal neurons, in some cases leading to improved visual function and restoration of light sensitivity [135, 140, 141]. Further investigations into the transplantation of hPSC-derived photoreceptors have demonstrated the use of immunodeficient mouse models to improve survival of hPSC-derived photoreceptors [136].

As the RPE provides essential support for photoreceptors, similar approaches for cell replacement have also been developed for RPE loss in retinal degenera-

tive diseases, often associated with the secondary loss of photoreceptors. hPSC-derived RPE has been utilized in the development of cell replacement strategies for diseases such as age-related macular degeneration [15, 21, 76, 107, 109, 114, 131]. In this capacity, the transplant of RPE cells has been accomplished by either subretinal injection as a cell suspension or as RPE sheet transplantation [142–151]. The latter approach may offer numerous advantages, as the cells retain their polarization and are arranged in a discrete monolayer, allowing better integration within the host retina. The success of the above-named transplantation strategies has paved the way for hPSC-derived RPE in clinical trials for AMD and Stargardt's disease, where transplanted cells were shown to improve visual acuity in patients, illustrating the ability of hPSCs to rescue visual defects in retinal degenerative diseases [14, 152].

Many of the cell replacement strategies developed to date have focused on the transplantation of RPE and/or photoreceptors due to their ease of differentiation and more limited need of these cells to extend neurites to form synaptic connections, which will likely make replacement efforts easier. However, the development of replacement strategies for inner retinal neurons such as RGCs is more complicated, largely due to their more elaborate nature and need to extend long axonal projections to form synaptic connections in the optic tectum [153–155]. As such, pharmacologic strategies to combat RGC degeneration have focused on early stages of the disease process where neuroprotection is feasible [63, 106]. The goal is both to improve RGC survival, as well as potentially regrow axons to reestablish central synaptic connections. Similar efforts have not been widely adopted yet for hPSC-derived RGCs, although early studies have demonstrated the ability of hPSC-derived RGCs to survive following intravitreal transplantation [80]. Further studies into the use of hPSC-derived RGCs are certainly warranted, as several recent reports have demonstrated the differentiation and enrichment of RGCs from hPSCs in vitro [63, 66, 67, 80, 82].

Efforts for cell replacement to date have often focused on the transplantation of a single type of retinal neuron. At late stages of retinal degeneration, other retinal neurons are often damaged and lost, leading to the need to replace multiple types of cells. Retinal organoids represent an exciting option for cell replacement at these late stages of retinal degeneration, as these organoids possess the relevant retinal cells pre-assembled into a stratified structure, and can serve as "mini-retinas" for replacement of retinal tissue [21, 23, 28–30, 32, 39, 63]. Early attempts at these strategies have recently been demonstrated in mouse models of retinitis pigmentosa, where retinal organoids were transplanted and retained transplants of retinal organoids in mice led to the retention of their three-dimensional architecture and formed presumptive synaptic connections with host bipolar cells [142]. Similar experiments have also been conducted in nonhuman primates, with the transplantation of hPSC-derived retinal organoids resulting in increased visual acuity [156].

2.8 Conclusions and Future Directions

Overall, research over the past several years has established hPSCs as a powerful tool for studying some of the earliest stages of human development that would otherwise remain inaccessible to investigation [19, 22, 23, 25, 27, 37]. This has encouraged the establishment of efficient differentiation protocols to generate all major cell types of the retina, including photoreceptors, RPE, and retinal ganglion cells [9, 20, 21, 30, 53, 55, 58, 61, 63, 70, 78, 81, 82, 113, 157, 158]. These hPSC-derived retinal cells have assisted in modeling retinal degenerative diseases, especially when generated from patients with inherited retinal dystrophies. For this purpose, patient-derived hPSCs have helped in understanding disease progression and mechanisms, and have subsequently enabled the identification of candidate neuroprotective factors to combat the degeneration of retinal neurons [11, 21, 39, 56, 57, 63, 76, 84, 98, 100, 103, 106, 115, 116, 128, 148]. However, these measures have limited utility at late-stage disease, where the loss of multiple retinal cell types is irreversible, resulting in severe loss of vision. As a source of cell replacement therapies, hPSC-derived retinal cells have been shown to integrate within the host retina, form synaptic connections as well as demonstrate functional rescue. Such strategies have been extensively studied in the context of RPE and photoreceptor degeneration [6, 7, 10, 39, 136, 141, 142, 149, 156, 159–161], and is finding application in current clinical trials in AMD and Stargardt's disease using hPSC-derived RPE [14, 152].

While tremendous progress has been made in the differentiation of retinal neurons from hPSCs [17, 19, 20, 22, 26, 53, 63, 69, 71, 81, 162], these cells often fail to fully differentiate into functionally relevant phenotypes which would better mimic the structure and functionality of the retina. Therefore, recent advances have led to the development of a three-dimensional approach to retinal differentiation, where hPSCs are directed to yield discrete populations closely analogous to the developing optic cup and eventually giving rise to a pseudostratified structure resembling the retina [21, 28–31, 33, 35–39]. With these advances, retinal organoids follow predicted stages of retinal development, and have led to enhanced differentiation and maturation of photoreceptors, facilitating the application of these approaches for studies of retinal development and pathogenesis in both normal and diseased states.

Patient-derived organoids may be best suited for assessing the effects of disease-related neurodegeneration on specific retinal cell types, as well as their interactions with each other. Currently, retinal organoids are likely better suited for studies of photoreceptor diseases, as photoreceptor development and maturation has been extensively characterized in retinal organoids, leading to rod-dominant retinal domains similar to peripheral regions of the retina [29, 30, 35, 38, 39]. Therefore, rod-cone dystrophies like retinitis pigmentosa, which begins as a peripheral retinal degeneration, can be most effectively modeled with retinal organoids, with the goal of developing neuroprotective strategies. Moreover, future efforts to characterize inner retinal neurons within retinal organoids will

help to model and develop therapies for RGC degeneration in optic neuropathies. In addition to studies of retinal development and disease, the most exciting feature of retinal organoids may be their ability to serve as a replacement for retinal tissue in severely degenerated retinas. The interconnected structure composed of multiple retinal neurons may facilitate integration and replacement of multiple cell types within the degenerated retina.

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