Chapter 1
Pluripotent Stem Cells to Model Degenerative Retinal Diseases: The RPE Perspective

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Abstract Pluripotent stem cell technology, including human-induced pluripotent stem cells (hiPSCs) and human embryonic stem cells (hESCs), has provided a suitable platform to investigate molecular and pathological alterations in an individual cell type using patient's own cells. Importantly, hiPSCs/hESCs are amenable to genome editing providing unique access to isogenic controls. Specifically, the ability to introduce disease-causing mutations in control (unaffected) and conversely correct disease-causing mutations in patient-derived hiPSCs has provided a powerful approach to clearly link the disease phenotype with a specific gene mutation. In fact, utilizing hiPSC/hESC and CRISPR technology has provided significant insight into the pathomechanism of several diseases. With regard to the eye, the use of hiPSCs/hESCs to study human retinal diseases is especially relevant to retinal pigment epithelium (RPE)-based disorders. This is because several studies have now consistently shown that hiPSC-RPE in culture displays key physical, gene expression and functional attributes of human RPE in vivo. In this book chapter, we will discuss the current utility, limitations, and plausible future approaches of pluripotent stem cell technology for the study of retinal degenerative diseases. Of note, although we will broadly summarize the significant advances made in modeling and studying several

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retinal diseases utilizing hiPSCs/hESCs, our specific focus will be on the utility of patient-derived hiPSCs for (1) establishment of human cell models and (2) molecular and pharmacological studies on patient-derived cell models of retinal degenerative diseases where RPE cellular defects play a major pathogenic role in disease development and progression.

Keywords Age-related macular degeneration · Choroidal neovascularization · Drusen · Human induced pluripotent stem cell (hiPSC) · hiPSC-based disease modeling · Retinal degenerative diseases · Retinitis pigmentosa · Retinal pigment epithelium

1.1 Overview of Retinal Degenerative Diseases with a Focus on RPE Cell Layer

Retinal degenerative diseases (RDDs) are, as a group, one of the leading causes of irreversible vision loss worldwide. These commonly include retinitis pigmentosa (RP), Leber congenital amaurosis (LCA), Stargardt disease, and age-related macular degeneration (AMD), affecting the central part of retina, the macula, responsible for central vision. Among these, AMD is the most common cause of blindness, affecting elderly individuals, over the age of 55. Apart from aging, epidemiological analyses have identified several genetic and environmental risk factors implicated in the onset and progression of AMD [1, 2]. Mendelian retinal disorders such as heterogenous RP, Stargardt disease, LCA, and Best disease are caused primarily by mutation of a single gene resulting in the absence of or a dysfunctional gene product [3–7]. Although RDDs differ phenotypically, the major retinal cell types primarily affected are photoreceptors, retinal pigment epithelium (RPE), and the underlying choroidal vessels. Furthermore, in a subset of inherited and age-related retinal degenerative diseases (e.g., Best disease, Sorsby's fundus dystrophy, AMD), primary molecular defects in the RPE cell layer in the eye have been implicated in disease development and progression [8–11]. This is not surprising, given that RPE cell health and function is essential for photoreceptor survival and thereby vision maintenance. A noninclusive list of RPE functions includes the absorption of light, exchange of biological materials between the photoreceptors and the choroid, the visual cycle, the processing of shed photoreceptor outer segments, and paracrine cellular communication [12, 13]. The absorption of light, specifically its energy, helps to protect the oxygen-rich retina from photo-oxidation. Light absorption occurs through melanin within the melanosomes and other RPE pigments that filter distinct wavelengths of light, protecting the macula from oxidative stress [14]. The hexagonal RPE forms tight junctions, with the cell layer classified as a tight epithelia, forming a diffusion-impermeable layer. As such, RPE must actively transport biological materials between their apically abutting photoreceptors and their basolateral choroid vasculature. This transport is critical to the balance of water, ions, and pH within the subretinal space. RPE facilitates the uptake of glucose and retinol

from the choroidal vasculature to the photoreceptors. The retinoids are the vehicle to transform light energy, through isomerization, to neurological signal giving us vision. Retinol is converted to 11-*cis*-retinal, within the RPE, prior to transport to the photoreceptors where it binds rhodopsin and is integrated into the visual cycle. Photoreceptors lack the isomerases necessary to regenerate 11-*cis*-retinal, which reside in the RPE and transport retinol from the choroidal vasculature, making them indispensable in the visual cycle. The recycling of photoreceptors is another important function of the RPE. Photo-oxidation and free radical generation of the mitochondrial-laden photoreceptors exposes their cellular contents to extensive oxidative damage. Removal of much of this damage is accomplished through outer segment shedding, in which photoreceptor outer segments (POS) containing damaged macromolecules are lost and phagocytosed by the RPE. RPE further degrades the POS contents while recycling retinoids for the visual cycle. Paracrine signaling between the photoreceptors and choroid is a highly coordinated process by the RPE. Among the notable proteins secreted from RPE with physiologic effects relevant to this dialog are pigment epithelium-derived factor (PEDF), vascular endothelial growth factor (VEGF), TIMP metallopeptidase inhibitor 3 (TIMP3), and EGF-containing fibulin extracellular matrix protein 1 (EFEMP1) [9, 12, 13]. PEDF is apically secreted by the RPE and is believed to be neuroprotective as well as anti-angiogenic. In juxtaposition, VEGF is basally secreted and promotes stabilization of the choroidal vasculature in addition to promoting angiogenesis [9, 15]. The proteins TIMP3 and EFEMP1 are involved in regulating extracellular matrix (ECM) turnover [16, 17], crucial for maintenance of the Bruch's membrane, and thereby diffusion and transport of biomolecules across the RPE and choroidal vasculature. Not surprisingly, these proteins have been implicated in macular degenerative diseases and are discussed within mechanisms of genetic macular degenerative diseases. In fact, defects in a number of these RPE cell functions (e.g., phagocytosis and degradation of POS, ECM turnover) have been implicated in both AMD and other RDDs that are caused by mutations in genes regulating these cellular processes (e.g., RP caused by *MERTK* mutations affecting POS phagocytosis [18–21], Doyne honeycomb retinal dystrophy (DHRD) caused by an *EFEMP1* mutation, a gene involved in ECM turnover [22, 23]).

1.1.1 Age-Related Macular Degeneration (AMD)

AMD is the leading cause of vision loss in the western world [24]. By the age of 75, approximately 30% of Americans are affected by the disease [25]. AMD exists in two forms, "dry" and "wet" AMD. Currently there is no treatment for "dry" or geographic atrophy, while "wet" AMD has multiple FDA-approved drugs [26]. Initially both forms of AMD present with a similar pathology are characterized by the appearance of intermediate-sized drusen within the macular region of the retina and thickening of the Bruch's membrane. The Bruch's membrane is a five-layer sandwich of connective tissue consisting of RPE basal lamina, an inner collagenous layer,

an elastic layer, an outer collagenous layer, and choriocapillaris basal lamina [9, 27]. The thickening of the Bruch's membrane in AMD consists of fibrous long-spacing collagen, which is geographically located between the RPE and the RPE basal lamina. Drusen are observed as whitish-yellow infiltrates by ophthalmoscopy and are composed of biological materials including protein and lipid or lipoproteinacous material. Within the structure of the retina, drusen reside between the basolateral side of the RPE and the Bruch's membrane; however, unlike the ordered collagenous thickening of the membrane, it appears as more random infiltrates. This positioning is likely critical to the role of drusen and membrane thickening in the development of AMD's pathology, disrupting the functional interaction between the RPE and the vasculature behind the retina. Drusen are characterized as either hard or soft, quantitatively by number and size by their size, <63 or <125 μm, respectively, and qualitatively by border appearance, distinct or fuzzy/diffuse [28]. A greater quantity and presentation of soft drusen was the best predictor of advanced AMD in the Beaver Dam 15-year follow-up study [29]. In the "dry" form of AMD, the appearance of drusen is followed by changes in macular retinal pigment, the atrophy of RPE and ultimately the loss of vision. In contrast, the wet form of AMD involves choroidal neovascularization (CNV) or the proliferation of vasculature from the choroid. While the nature of this event remains ill-defined in AMD progression, stimulation of RPE by complement components C3 and C5, common residents of drusen [30] promoted VEGF secretion from the RPE upon acute exposure in a murine model [30]. This suggests the appearance of drusen in early AMD predisposes the choroid to neovascularization while reinforcing the paracrine signaling balance between the RPE and choroid, here in the promotion of angiogenesis [30, 31]. Genetically an increased susceptibility to AMD has been linked to polymorphisms in genes involved in the complement immune response. The genes implicated include Complement factor H (*CFH*), *C3*, Complement factor I (*CFI*), and Complement Factor B (*CFB*) [32], suggesting a disrupted and/or dysregulated immune response may contribute to the pathology. Polymorphisms within the Age-Related Maculopathy Susceptibility-2 (*ARMS2*) gene are linked to increased susceptibility to AMD [33], though the function of this protein in the disease pathology remains undefined.

While no single mutation is responsible for the development of AMD, there are other forms of late-onset retinal degeneration that do result from single-point mutations. Specifically Sorsby's Fundus Dystrophy (SFD) with a mutation in TIMP3 [34] and Doyne's Honeycomb Retinal Dystrophy/Malattia Leventinesse (DHRD/ML) with a mutation in *EFEMP1* [35], both of which are involved in ECM remodeling, is secreted from the RPE. Likewise, mutations in *C1QTNF5* gene (*CTRP5*), an RPE-secreted protein, which is a constituent of Bruch's membrane leads to late-onset retinal degeneration (L-ORD) [36, 37]. Interestingly these diseases appear to share a significant phenotypic overlap, the development of drusen, integral to progression of the pathology. Despite their inheritance through genetic mutation, these diseases have a relatively late-onset, though earlier than that of AMD, suggesting the development of the pathology is time-dependent despite the immediate expression of a mutated gene product. These characteristics make these diseases plausible surrogates to study the complex time-dependent development of AMD.

1.1.2 Sorsby's Fundus Dystrophy (SFD)

SFD is an autosomal dominant macular degenerative disease, which begins to affect patients between their third and fifth decades of life [38]. The earliest symptom is usually a loss of night vision. Patients may then experience rapid central vision loss followed by peripheral vision loss [39]. The pathology of the disease can be similar to that of wet AMD with neovascularization observed in SFD patients, which may lead to the acute vision loss. Alternatively, a slowed progression with peripheral vision loss parallels the pathophysiology of dry AMD with loss of RPE in distinct areas. Common to both forms is the appearance of peripheral drusen between the RPE and the Bruch's membrane. Unlike AMD where a number of mutations across multiple alleles increase the probability of its development, mutation of a single protein, TIMP3, has been defined as causal in SFD [34]. A number of distinct mutations within TIMP3 have been described to be responsible for the pathology of SFD, most of which cause a missense mutation introducing an additional cysteine residue and all of them clustering around exon 5 of the mRNA message [40]. The mutations lie in the c-terminal region of TIMP3, which has been ascribed the function of inhibition of matrix-metalloproteases (MMPs) [41]. The additional cysteine residues and observed TIMP3 aggregates in SFD patient's led investigators to hypothesize that additional disulfide bridges between TIMP3 molecules may drive the aggregation of protein affecting its interaction and inhibition of MMPs. In agreement, a defining pathologic marker of the disease is a thickening of the ECM adjacent to the Bruch's membrane, suggesting this may indeed affect regulation of ECM remodeling machinery. However, functional analysis of these mutations gave varied results with respect to the ability of the mutated protein to inhibit MMP2 despite the increased propensity of the mutants to form oligomers [16]. It is currently postulated that the oligomers of TIMP3 instead disrupted TIMP3 turnover, leading to their observation of the extreme thickening of the Bruch's membrane. Notably, as mentioned previously, similar to AMD, SFD patients present drusen accumulation between the RPE and the Bruch's membrane preceding the retinal atrophy of the RPE and photoreceptors. Furthermore, TIMP3 protein is a prominent constituent of drusen deposits in both AMD and SFD [10, 42], and rare variants in TIMP3 have recently been linked to AMD development [43].

1.1.3 Doyne's Honeycomb Retinal Dystrophy (DHRD/ML)

DHRD/ML is a macular degenerative disease pathologically identified by the appearance of yellowish-white drusen, RPE atrophy, and neovascularization preceding the loss of vision [44, 45]. Patient vision loss generally has onset in the fourth decade of life. DHRD/ML results from the missense mutation (R345W) in *EFEMP1* gene [35]. EFEMP1 is a secreted protein that has been shown to regulate the activity of multiple MMPs (e.g., MMP-2, MMP-9) involved in ECM turnover [23]. The R345W mutation in *EFEMP1* has been demonstrated to inhibit the proper

folding and secretion of the protein [17] and initiating the unfolded protein response (UPR) within RPE [46]. Although the persistent stimulation of the UPR promotes apoptotic cell death, the exact mechanism of how R345W mutation in *EFEMP1* leads to the progression of the disease remains ill-defined. It has however been postulated that *EFEMP1* mutation promotes the DHRD/ML pathology in a dominant negative fashion. The dominant negative action of the mutation is supported by the R345W *EFEMP1* knock-in mice developing pathological markers of the disease, including drusen development at the Bruch's membrane [47], while knock out mice are unaffected with respect to macular health [48]. Recent studies utilizing R345W *EFEMP1* knock-in mice and overexpression of mutant *EFEMP1* in cultured RPE cells, ARPE19, and human fetal RPE have implicated a causal role for C3 activation in the formation of drusen/basal deposits in DHRD, due to impaired ECM turnover [23, 47, 49]. Interestingly, EFEMP1 has also been shown to be a binding partner of TIMP3 [17], the gene causal in SFD, and TIMP3 and EFEMP1 colocalize in the drusen deposits underlying DHRD patients [45]. Furthermore, highlighting a plausible common underlying pathological progression between these distinct diseases; AMD, SFD, and DHRD, similar to TIMP3, EFEMP1 is also found within the drusen deposits underlying AMD patients [22].

1.1.4 Late-Onset Retinal Degeneration

Late-onset retinal degeneration (L-ORD) also sometimes referred to as late-onset macular degeneration (L-ORMD) [50, 51] is a rare autosomal dominant retinal dystrophy primarily affecting the interior segment and retina [52, 53]. A single founder mutation (Ser163Arg) in Complement 1q Tumor Necrosis Factor 5 gene (*C1QTNF5*, previously called *CTRP5*) on chromosome 11 is the causative mutation in L-ORD [36, 37]. *C1QTNF5* is commonly expressed in RPE and ciliary epithelium and is comprised of three domains; a signal peptide at the N-terminus, a short collagen repeat (Gly-X-Y), and a globular complement gC1q domain at the C-terminus and is proposed to function in the trimerization and folding of collagen [36, 51]. Affected individuals display normal visual acuity and fundus examination in stage 1 (ages 0–40); however, some may develop long anterior zonular fibers, iris atrophy, and secondary glaucoma [37, 54, 55]. Patients exhibit disease-like symptoms in stage 2 (fourth to fifth decade of life) exhibiting abnormalities in adaptation from light to dark conditions, perimacular yellow spotting, and midperipheral pigmentation in fundus photographic examination [53, 56]. In stage 3 (sixth decade of life), the patients develop CNV and decline in rod and cone function with sudden loss of visual acuity. At stages 2 and 3 with disease features such as subretinal basal deposits and CNV, L-ORD phenotypically resembles other inherited macular dystrophies including SFD, DHRD, as well as AMD [53]. However, it differs from AMD in terms of inheritance pattern and severity of the extension of sub-RPE deposits and atrophy causing decline of both central and peripheral vision [36, 53]. It varies from SFD and DHRD in terms of disease-causing mutations, later-onset yellow spotting

in fundus examination and display of abnormal dark adaptation in L-ORD compared to SFD [36, 53] and variability in the geographic confinement of drusen-like deposits and retinal atrophy in L-ORD compared to DHRD [57, 58]. Despite the differences in the onset and disease severity between L-ORD, SFD, and DHRD, the commonalities in the disease phenotype suggests similar disease pathogenesis among them. In fact, similar to DHRD [46], a study by Shu et al. has implicated endoplasmic reticulum (ER) stress in L-ORD pathology by showing misfolding of mutant *C1QTNF5* and its accumulation in the ER [51].

1.1.5 Best Disease (BD)

BD caused by mutations in Bestrophin-1 gene (*BEST1*) is a childhood-onset inherited dominant form of macular dystrophy characterized by subretinal macular deposition of round or oval yellowish vitelliform lesions [6, 59, 60]. Of note, apart from Best disease, mutations in BEST1 can lead to adult-onset vitelliform macular dystrophy (AVMD) [61], autosomal recessive bestrophinopathy (ARB) [62], autosomal dominant vitreoretinochoroidopathy (ADVIRC) [63], and retinitis pigmentosa 50 (RP50) [64]. Importantly, highlighting a role of RPE dysfunction in BD pathology, BEST1 in the eye, is exclusively expressed within the RPE monolayer. Although the disease mechanism by which mutations in BEST1 lead to BD pathology are still under investigation, several studies have now shown a role of BEST1 in regulating calcium and chloride ions in the RPE cell layer [65–68]. Furthermore, a defect in structural contact between the RPE layer and photoreceptor outer segment (POS) and POS handling [68–70] has been implicated in the pathology of BD. This hypothesis is consistent with the abnormal accumulation of autofluorescent material, lipofuscin, (undigested breakdown products of POS) in the retina, an RPE layer of the affected patient eyes, and consequent photoreceptor degeneration and decline in central vision [6, 71, 72].

1.1.6 Retinitis Pigmentosa

With worldwide prevalence ranging from 1:3000 to 1:7000, RP is the most common hereditary degenerative disorder of the retina. It predominantly affects the photoreceptors leading to rod and cone cell death [73, 74]. Common symptoms include night blindness, decline in electroretinogram responses, gradual loss of peripheral vision subsequently leading to irreversible vision loss. With disease-causing mutations identified in more than 85 genes [75, 76], RP can be inherited in autosomal dominant, autosomal recessive, and X-linked pattern. Apart from photoreceptor-specific cellular defects, disease-causing mutations in RPE-specific genes are also known to contribute to RP development. For example, mutations in the genes involved in visual cycle in the RPE cells have been associated with RP. These include autosomal

recessive forms of RP caused by mutations in membrane-type frizzled-related protein (*MFRP*) [77, 78], Mer tyrosine kinase receptor (*MERTK*) [79], and cellular retinaldehyde-binding protein (*CRALBP*) [80]. MFRP is a type II transmembrane domain protein shown to localize apically in RPE microvilli with mutant form leading to defective RPE morphology, cell junctions, and loss of microvilli [81]. In the RPE layer, MERTK regulates the recognition and internalization of POS during phagocytosis, and defective MERTK leads to retinal degeneration via failure of POS phagocytosis [19, 20]. Similarly, CRALBP is present in RPE and Muller cells of the retina and serves as a retinoid carrier involved in the oxidation of 11-*cis*-retinol to 11-*cis*-retinal [82, 83] and is critical for visual cycle regulation and thereby vision. Other RPE-specific visual cycle genes/proteins linked with RP development include lecithin retinol acyltransferase (*LRAT*) and RPE-specific 65 kDa (*RPE*65). Specifically, mutations in *LRAT* and *RPE65* have been reported to account for early-onset forms of RP [84, 85].

1.2 Animal Models of Retinal Degenerative Diseases

The generation of murine models, either knock-in or knockout, has been common practice to the study of genetic diseases. In the study of RDDs, murine models have been generated incorporating known environmental stressors or targeting various genes associated with the disease, with varied results in terms of the replication of disease pathology.

Early murine models of AMD focused on environmental factors contributing to AMD, namely the correlation to obesity and metabolic disease [86, 87]. In these models that were fed a high-fat diet, both age and high-fat diet correlated with an increased thickness of the Bruch's membrane in addition to deposits described as electron lucent particles. However, the thickening of the membrane was not the organized collagenous network observed in aging humans and the particles observed at the Bruch's membrane, and RPE did not contain cholesterol. Efforts to induce hyperlipidemia and elevated cholesterol through gene ablation or transgenic mice have also been used to examine their effect on the development of AMD. The ablation of the apolipoprotein E (*APOE*) gene, a lipid carrier in the blood, resulted in increased thickness of the Bruch's membrane with the appearance of membrane-bound materials [86]. Deletion of the low-density lipoprotein (LDL) receptor again resulted in thickening of the Bruch's membrane with increased lipid deposition at the membrane [88]. Differing results were observed in mice with a mutant form of very low-density lipoprotein (VLDL) receptor gene where an early CNV event occurs in mice as young as 2 weeks [89]. This rapid onset of CNV is seemingly in opposition to CNV in late "wet" AMD.

The role of oxidative stress has been investigated through the deletion of the antioxidant gene including superoxide dismutase 1 (*SOD1*). Along with thickening of the Bruch's membrane, these mice also developed drusen and CNV [31]. These pathological features were apparent only in aged mice, in agreement with the idea

that the cumulative insult of oxidative stress promotes AMD. Drusen from these mice contained C5, consistent with human drusen composition [31]. Cigarette smoking, along with being the highest correlative environmental factor with the progression of AMD, promotes oxidative stress. It follows that a smoking mouse model, in which mice were exposed to chronic cigarette smoke, showed increased accumulation of complement factors, C3a, C5, and the membrane attack complex (MAC) C5–9 in the Bruch's membrane relative to control mice [90]. Increases in oxidative stress within the RPE, cellular apoptosis, and thickening of the Bruch's membrane were also observed in response to cigarette smoke exposure in mice [91].

The complement system is well represented within the gene loci associated with the AMD pathology (Sect. 1.1.1). The deposition of C3 and C5 at the Bruch's membrane in the disease pathology also suggests an active role for the complement system in AMD's pathology. It follows that many murine models targeting these genes have been generated to study the disease. Within the retina and RPE/choroid complex of mice, the classical complement factors C1qb, C1r, C1s, C2, and C4 were observed to be constitutively expressed [92, 93]. The alternative pathway components CFH, CFB, C3, and C5 were also detected in these tissues while those in the mannose-binding lectin pathway were extremely low and/or undetectable. The expression of many complement components in the retina and choroid/RPE suggested that murine genetic models of AMD, through manipulation of the complement genes, could plausibly yield mechanistic insight about the disease.

The mutation Y204H within Complement factor H (*CFH*) is associated with a sevenfold increase in the development of AMD in humans [94]. Transgenic mice have been generated that harbor both Y204H point mutation and total gene ablation. The deletion of *CFH* resulted in AMD-like accumulation of drusen, which included C3, and photoreceptor atrophy in aged mice [95]. The accumulation of C3 was also observed in the proper physiologic region, between the basolateral side of the RPE and the Bruch's membrane. The point mutant CFH Y402H mice also displayed an increase in drusen, the thickening of the Bruch's membrane, and C3 accumulation; however, the disease pathology failed to advance to the level of photoreceptor loss [96]. While the knockout of either C3 or C5 proteins has not been reported to describe their effect on the progression of AMD, the reciprocal mice, the knockouts of the C3aR and C5aR receptor proteins have been generated [30]. In the receptor knockout study, the authors hypothesized that C3a and C5a accumulation drives increased VEGF secretion that promotes CNV, given that drusen development and complement factor deposition precede CNV. This hypothesis was based on a prior publication by Ambati et al. in which knockout of Ccr-2 and Ccl2, also known as monocyte chemoattractant protein 1 (MCP1) and its receptor, resulted increased sub-RPE C5a accumulation, by the lack of its removal by the immune system, with a coordinate stimulation of VEGF secretion by the RPE [97]. After initially demonstrating that C5 stimulated VEGF secretion from RPE, laser ablation was used to promote CNV, which was suppressed in the MCP1 receptor knockout mice but not completely abolished [30]. Taken together, these studies support a role for the complement system in the progression of AMD, likely not only in its ability to induce cell death through MAC, but it may also be integral to the promotion of angiogenesis

in CNV. This distinction would be relevant to therapeutic intervention against the more damaging form of "wet" AMD. There are currently no murine models of AMD with the manipulation of CFI or CFB, wherein mutations of both are reported to correlate with an increased susceptibility to develop AMD [98, 99].

Using the knowledge of mutations in causative genes identified in inherited late-onset macular dystrophies, murine models for SFD, DHRD, and L-ORD have been developed. In the case of SFD, a knock-in model was created carrying the TIMP3 Ser156Cys mutation [100]. Intriguingly, TIMP3 knock-in mice do not display any pathological manifestations of the human disease. In addition, no formation of basal deposits or ECM thickening was documented in these mice [100]. A knock-in mice model of DHRD with EFEMP1 R345W mutation [47] show increased basal deposits directly linking C3 activation to the formation of basal deposits [49]. A few different murine models of L-ORD [50, 101–103] (heterozygous knock-in mice *Ctrp5*$^{+/-}$, (hC1QTNF5(S163R)-HA) *C1qtnf5*$^{+/\mathrm{Ser163Arg}}$, *Ctrp5*$^{+/-}$;*rd*^{8}/*rd*^{8}) have also been established and show a range of key disease-related features of L-ORD and AMD commonly observed in humans [36, 56], including abnormalities in dark adaptation, increased autofluorescent accumulation in the retina, increased abundance in sub-RPE drusen-like deposits, retinal degeneration and abnormalities in Bruch's membrane, and significant loss of photoreceptors cells. In fact, using a recombinant adeno-associated viral (AAV) vector approach, Dinculescu et al. generated a mouse model (hC1QTNF5(S163R)-HA) expressing the *Ctrp5/C1QTNF5* gene driven by RPE-specific BEST1 promoter to investigate the in vivo consequences of the disease-causing mutation in specific to the RPE [103]. This L-ORD mouse model displayed abnormal accumulation and distribution of the mutant gene within the RPE cells leading to sub-RPE deposits resembling *EFEMP1* knock-in mice [44, 47]. However, deposits of (hC1QTNF5(S163R)-HA) mice lacked positive staining for lipids, a known component of sub-RPE deposit in L-ORD patients [56, 104] while *C1qtnf5*$^{+/\mathrm{Ser163Arg}}$ mice failed to manifest the L-ORD phenotype throughout its life span [50]. Mouse models both knock-in (*Best1*$^{+/W93C}$, *Best1*$^{W93C/W93C}$) and knockout (*Best1*$^{-/-}$) have also been used to investigate Best disease pathophysiology in vivo [67, 105, 106]. Zhang et al. generated a knock-in mouse carrying the disease-causing mutation W93C in *BEST1* [67]. This mouse model harbored several of the BD-related features including reduced light peak, lipofuscin accumulation in the RPE, and serous/debris-filled retinal detachment. They also noticed disrupted photoreceptor outer segments suggesting partial impairment of POS phagocytosis by RPE in BD [67]. However, other Best1 knockout mouse models failed to recapitulate the ocular phenotypes of BD. Similarly, contradictory results of functional tests evaluating BEST1 function (Cl^- channel, Ca^{2+}-activated Cl^- channel (CaCC), volume regulation) were found in the distinct BD mouse models [67, 105, 106]. Several groups have also generated transgenic mice models to investigate the causative role of RPE-specific genes in the pathogenesis of RP. For example, the Royal College of Surgeons (RCS) rats harboring the MERTK mutation associated with early onset of RP [107] are a well-studied model of retinal dystrophy [18, 108]. Retinal degeneration in the RCS rat is associated with defects in POS phagocytosis by the RPE leading to accumulation of phagocytosed OS with alterations in OS length eventually affecting the photoreceptors [108–110]. A similar degenerative retinal

phenotype has also been reported in the *mer*^{kd} mice corroborating the causative role of MERTK mutations in RP [19]. A preclinical mouse model of RP, *Mfrp*^{rd6}/*Mfrp*^{rd6} [111, 112], that shows progressive degeneration of the retina and photoreceptors is commonly used to test in vitro RP-related therapies [81, 113]. Of note, although the RP mouse models have provided significant insights into the human disease pathomechanism, contrasting observations have been made in MFRP mutant mice models and MFRP patients [81, 114, 115]. For example, *Mfrp*^{rd6}/*Mfrp*^{rd6} and *Mfrp*174delG mice models displayed an increased number of RPE microvilli with no alterations in their length, while the shortened and reduced number of microvilli was reported by Won et al. in the mouse model *Mfrp*^{rd6}/*Mfrp*^{rd6} [115]. In contrast, electron microscopy demonstrated loss of apical RPE microvilli in patients with mutant MFRP [81].

Apart from extensively used murine models, other animal models including those derived from rats, pigs, rabbits, and non-human primates have proved to be invaluable in procurement of our current knowledge about the histological features and pathophysiology of specific RDDs. However, apart from non-human primates, the other available animal models display considerable differences with respect to genetic background and physiology within the human retina. For example, a major disadvantage of rodent models is the complete lack of macula, which is the site of disease manifestation, and hence, they fail to recapitulate the AMD disease phenotype as seen in humans. On the other hand, non-human primates anatomically resembling the human retina with the presence of a macula demonstrate early to intermediate features of AMD [116]. However, non-human primate models possess certain obstacles such as difficulty in genetic manipulation, expensive maintenance of non-human primates and slow progression of the disease that does not correlate with that of humans [116].

Other alternatives that have been utilized to interrogate specific RDDs include histopathological examination of the human cadaver eyes and mammalian overexpression systems, including those utilizing immortalized RPE cell lines (e.g., ARPE-19 [117]), and primary RPE cells in culture (e.g., hfRPE and porcine RPE [118–120]). Although these approaches have generated important information about the end-stage pathology and RPE physiology, an optimal platform for understanding the mechanisms behind degenerative diseases of the retina would allow observations of the progression of the disease, i.e., affected cells/tissues from the living human eye progressing through the disease. This is especially relevant, given that postmortem samples are rarely available and are at the end stage of the disease and thus provide no insight into the early events that were causal in the disease development.

1.3 The Pluripotent Stem Cell Technology and Its Utility for Studying Retinal/RPE-Based Disorders

Access to biological samples from human retina and choroid for cellular and molecular studies had not been possible until the advent of the hiPSC technology. The description of reprogramming factors by Yamanaka and Thomson groups [121, 122]

made it possible to generate pluripotent stem cells with the ability to differentiate into any of the three germ line lineages and many of their mature cell types. Since then iPSCs derived from patients have been used as a platform to investigate disease pathophysiology and screen for drugs and possible therapeutic approaches. hiPSCs have been generated from a range of sources including fibroblasts, keratinocytes, lymphocytes, core blood cells, adipocytes, and T cells [123–129]. It is noteworthy that several studies have now demonstrated that (1) retinogenesis in a hiPSC-derived model system follows the time course and sequence of retinal development in vivo [130], (2) major cell type(s) of the retina can be consistently differentiated from hiPSCs [127], and (3) hiPSC-derived retinal cells, including RPE, display several important physical and functional attributes akin to their in vivo counterpart. Furthermore, we and others have demonstrated the utility of hiPSCs for studying (1) human retinogenesis and retinal developmental disorders [22, 69, 131] and (2) retinal degenerative diseases like Stargardt disease, BD, glaucoma, and AMD [69, 123, 132–134]. With regard to disease modeling, mechanistic, and pharmacological studies, hiPSC-derived cell models of inherited RDDs, like BD, SFD, DHRD, ADRD, L-ORD, RP, and AMD have clearly established the utility of hiPSC-derived disease models for studying and pharmacologically targeting retinal diseases, including those caused by RPE dysfunction [20, 22, 69, 135–138].

1.3.1 hiPSC Models to Study AMD and Related Retinal Dystrophies (SFD, DHRD, L-ORD)

As mentioned previously, AMD and related retinal dystrophies (SFD, DHRD/ML, and L-ORD) are characterized by formation of lipid-protein-rich basal deposits (drusen), thickening of Bruch's membrane and eventually loss of RPE/photoreceptor layers [9, 139, 140]. Furthermore, a subset of patients with each disorder (AMD, SFD, DHRD/ML, and L-ORD) can develop vision loss due to CNV where in choroidal vasculature grows into the subretinal space [8, 53, 141, 142]. Although the CNV phenotype can be treated in patients (e.g., AMD, SFD [26, 143]), overall the lack of knowledge of molecular and cellular events occurring during the early stages of these disorders, which are causal in disease pathology (e.g., drusen formation), has been detrimental to our ability to develop rational drug therapies.

In a landmark study, Saini et al. utilized hiPSC-RPE derived from AMD patients to (1) investigate the early molecular events in the disease development and (2) test the efficacy of specific drugs in modulating the effected cellular pathway [137]. Specifically, in this study, researchers generated hiPSC-RPE from patients diagnosed with AMD who were homozygous/heterozygous for *ARMS2*/*HTRA1* and age-matched unaffected controls that showed protective alleles at both loci. Although no differences in baseline RPE characteristics, including expression pattern of RPE signature genes and transepithelial resistance, were found in control vs. AMD hiPSC-RPE, AMD hiPSC-RPE displayed increased expression of drusen components (APOE, amyloid-beta or Aβ) and complement pathway genes [137].

Furthermore, AMD hiPSC-RPE compared to control hiPSC-RPE displayed increased basal secretion of complement protein (C3). Notably, utilizing AMD hiPSC-RPE cultures in long-term experiments spanning 3–12 weeks, the authors demonstrated the ability of a specific drug, nicotinamide (NAM), in suppressing the expression of genes and/or secretion of proteins associated with drusen formation (APOE, APOJ, VEGF-A) and complement pathway (CFH and C3) [137].

In the first study to mimic the drusen phenotype in patient-derived hiPSC-RPE cultures, Galloway et al. utilized hiPSC-RPE from patients with SFD, DHRD, and autosomal dominant radial drusen (ADRD) [22]. Specifically, by utilizing the prolonged culture life of hiPSC-RPE cells and "aging" control (hiPSC-derived from unaffected family members and/or isogenic gene-corrected hiPSC line) and SFD, DHRD, and ADRD hiPSC-RPE cultures (≥90 days in culture), the authors were able to show the presence of basal deposits in both control and patient-derived hiPSC-RPE monocultures. Importantly, basal deposits in patient-derived hiPSC-RPE culture were significantly more than control hiPSC-RPE cultures, present beneath the COL4-positive basement membrane, and demonstrated the presence of several drusen-characteristic proteins, APOE, TIMP3, and EFEMP1. Furthermore, consistent with observed ECM alterations in SFD, DHRD, and ADRD eyes, patient-derived hiPSC-RPE cultures compared to control hiPSC-RPE showed increased accumulation of a specific ECM protein, COL4. Ultimately by using hiPSC-RPE monocultures, from (1) patients with known genetic defect affecting RPE cells (*TIMP3* in SFD; *EFEMP1* in DHRD) and (2) patients with an unidentified genetic defect (ADRD), this study confirmed the causal role of RPE defects in instigating two specific disease hallmarks of AMD and related macular dystrophies, drusen formation, and ECM accumulation.

Gamal et al. utilized a combination of hiPSC-based disease modeling and tissue-on-a-chip approaches to model the events in healthy vs. diseased (L-ORD) RPE following an electrical insult mimicking damage to the RPE cell [144]. Specifically, hiPSC-RPE derived from an affected patient with L-ORD and an unaffected sibling were grown as a monolayer on Electric Cell-Substrate Impedance Sensing (ECIS) microelectrode arrays [145, 146]. The tissue-on-chip approach was then used to investigate the ability of control vs. L-ORD hiPSC-RPE to repair following damage induced by electric wound. Notably, L-ORD hiPSC-RPE demonstrated an impaired rate of wound healing by displaying a reduced rate of migration and dissimilar migration patterns. Of note, for effective cell-substrate attachment and release, a cell line should display optimal migration rate with intermediate adhesion levels [147]. The authors concluded that the reduced migration rates in L-ORD hiPSC-RPE could be accounted for by its stronger adhesion properties to the cell-substrate compared to the control hiPSC-RPE.

Chang et al. generated hiPSCs from the T cells of patients with intermediate and advanced dry AMD and further differentiated them into RPE for use in disease modeling and pharmacological studies [128]. Although, AMD hiPSC-RPE and control hiPSC-RPE showed similar expression of RPE-specific markers such as RLBP1, RPE65, MITF, and PAX6, AMD hiPSC-RPE displayed higher accumulation of endogenous reactive oxygen species (ROS). The increased levels of ROS in AMD

hiPSC-RPE cultures were further exacerbated by treatment with H_2O_2. Interestingly, screening of several candidate drugs demonstrated that treatment with curcumin leads to significant reduction in ROS levels in AMD hiPSC-RPE cells. This is an important finding given the fact that oxidative stress has been implicated to cause RPE cell damage in AMD [128].

Yang et al. generated hiPSC-RPE from AMD patients and utilized Bis-retinoid N-retinyl-N-retinylidene ethanolamine (A2E) and blue light exposure to "age" these cells in culture. Interestingly, in comparison to control hiPSC-RPE derived from individuals with homozygous protective haplotype (G–Wt–G; G–Wt–G) for AMD susceptibility, AMD hiPSC-RPE derived from patients with known AMD risk alleles (heterozygous T-in/del-A; G–Wt–G, and homozygous T-in/del-A; T-in/del-A) and showed impaired SOD2 activity accompanied with elevated levels of reactive oxygen species (ROS), thus providing a potential link between oxidative stress and AMD development in individuals harboring the AMD risk alleles (T-in/del-A; G–Wt–G, T-in/del-A; T-in/del-A) [148]. Another AMD-related risk allele identified by genome-wide association study (GWAS) is the complement H factor (*CFH*), and polymorphisms in the *CFH* gene have been strongly linked to AMD pathogenesis via the activation of complement system [149, 150]. Hallam et al. generated hiPSC-RPE from patients harboring the Y402H mutation in the *CFH* gene with varying disease severity. Notably, in the absence of any extrinsic stressors and consistent with AMD disease pathology, patient-derived hiPSC-RPE showed presence of drusen-like deposits that contained known drusen proteins, APOE and C5b-9. Furthermore, the authors reported increased susceptibly to oxidative stress and defective autophagy in AMD hiPSC-RPE cells. In addition, this study also tested the efficacy of treating patient hiPSC-RPE with UV light as a possible treatment therapy. Remarkably, UV light elicited a different response in the low- and high-risk AMD hiPSC-RPE as assessed by the functional and structural characteristics of RPE cells after UV treatment [136]. Also, assessing the role of oxidative stress in AMD, Garcia et al. utilized in vitro modeling of cellular events associated with chronic oxidative stress related to AMD in RPE in both hiPSC and hESC RPE cells [151]. Specifically, chronic exposure to paraquat, activated the NRF2-KEAP1 pathway following induction of specific effectors during the early and late-stage responses, including upregulation of p21, alterations in the microRNA levels (has-miR-146a, has-miR-29a, has-miR-144, has-miR-200a, has-miR-21, has-miR-27b) and identification of Ai-1, an activator with protective role against oxidative stress. Overall, this study successfully illustrated the antioxidant responses and the protective role of the NRF2 pathway in human RPE cells.

To investigate the pathophysiological pathways contributing toward mitochondrial dysfunction in AMD, Golestaneh et al. derived hiPSC-RPE from two AMD patients with abnormal *ARMS2*/*HTRA1* alleles and one AMD patient with normal *ARMS2*/*HTRA1* and protective factor B alleles. In accordance with increased susceptibility of AMD hiPSC-RPE to oxidative stress [148], Golestaneh et al. reported similar observations of increased ROS levels and failure to increase SOD2 expression under conditions of oxidative stress in AMD hiPSC-RPE along with ultrastructural damage and dysfunction of mitochondria. Given that peroxisome

proliferator-activated receptor-gamma coactivator (*PGC*)-1α is involved in mitochondrial biogenesis [152] and silent information regulator T1 (*SIRT1*) is a known regulator of *PGC-1α* [153], the authors sought to further gain insight into the underlying mechanisms responsible for mitochondrial dysfunction in AMD hiPSC-RPE. Notably, AMD hiPSC-RPE displayed reduced expression of PGC-1α and SIRT1 protein levels possibly due to AMPK inactivation, thus implicating the involvement of AMPK/PGC-1α/SIRT1 pathway in AMD pathogenesis.

1.3.2 hiPSC Models of RP

The heterogeneous nature of RP and involvement of both photoreceptor and RPE cells in the disease pathology has made it difficult to identify the impact of disease-causing mutations on individual cell type (RPE vs. photoreceptors) and their consequences for disease development in vivo. Furthermore, available animal models of RP do not fully recapitulate the heterogeneous RP phenotype observed in human patients that develops partially due to differences in genetic make-up of affected individuals [154–157]. These limitations make hiPSC technology particularly attractive to study RP as the disease pathomechanism can be interrogated in an individual cell type (photoreceptor, RPE) using patient's own cells.

Most hiPSC models of RP have typically been developed using a two-dimensional approach by differentiation of patient-derived hiPSCs into either RPE or photoreceptors depending on the cell types affected by the disease-causing gene. The Takahashi group was one of the first groups to successfully generate multiple patient-derived hiPSC lines from five distinct RP patients carrying mutations in *RP1* (721Lfs722X), *RP9* (H137L), *PRPH2* (W316G), or *RHO* (G188R) genes [138, 158]. Given that these mutations affect photoreceptor cells, hiPSCs in this study were differentiated into photoreceptor cells. Furthermore, electrophysiological and gene expression analysis confirmed the functional and molecular characteristics of hiPSC-photoreceptors. Further analysis of patient-derived hiPSC photoreceptors showed elevated oxidative stress and ER stress markers with the selective loss of mature rod photoreceptor cells, whereas cone photoreceptors remained unaffected [138, 158]. Similar observations were made in hiPSC photoreceptors derived from an RP patient carrying a different *RHO* mutation (E181K) [159]. To further corroborate causal role of mutant *RHO* gene on development of the RP-disease phenotype in this study, the authors introduced the mutant *RHO* gene harboring E181K mutation in control hiPSCs, with similar results. Remarkably, the authors also reverted the observed disease phenotype in hiPSC-photoreceptors by correcting the mutation using helper-dependent adenoviral vector [159]. In addition, using hiPSC photoreceptors for drug screening studies, Yoshida et al. demonstrated the protective effect of rapamycin, 5-aminoimidazole-4-carboxyamide ribonucleoside (AICAR), Nuclear Quality Assurance-1 (NQDI-1), and salubrinal on rod photoreceptor cell survival [159].

Tucker et al. demonstrated a novel mutation in a newly identified RP gene encoding male-germ cell-associated kinase using an array of sequencing techniques and

hiPSC-derived retinal cells [160]. Using a similar approach, applying a combination of sequencing and molecular studies on hiPSC-derived retinal precursor cells, Tucker et al. also identified disease-causing mutations in *USH2A* gene and showed that disease-causing *USH2A* variants lead to protein misfolding and ER stress [123].

A similar approach of using patient-specific hiPSC lines has been utilized to study and pharmacologically target the RPE-disease phenotype in RP patients. Schwartz et al. generated hiPSCs from X-linked RP patients carrying the nonsense mutation c.519C>T (p.R120X) in *RP2* gene and differentiated them into RPE cells [161]. The RP2 protein was not detectable in *RP2* R120X patient-derived hiPSC-RPE cells. In conjunction, *RP2* R120X hiPSC-RPE showed defects in Intraflagellar Transport 20 (IFT20) localization, Golgi cohesion, and G protein beta subunit (Gβ1) trafficking. Remarkably, using translational read-through-inducing drugs (TRIDs), the authors partially recovered RP2 protein and consequently reversed the phenotypic abnormalities observed in *R120X* hiPSC-RPE cells [161]. Using a similar approach and utilizing TRIDs on patient-derived hiPSC-RPE from an individual having RP due to the presence of a nonsense variant of *MERTK* gene, Ramsden et al. were also effective in partially restoring the affected function of MERTK, recognition, and internalization of photoreceptor outer segments (POS), in patient-derived hiPSC-derived RPE cells [21].

Li et al. developed an hiPSC-RPE cell model of an autosomal recessive form of RP with mutations in the Membrane Frizzled-Related Protein (*MFRP*) gene that displayed defects in RPE cell pigmentation, morphology, and tight junction formation [81]. Notably, utilizing a gene therapy approach, the authors reversed the disease-specific phenotype in patient-derived hiPSC-RPE cells by AAV8-mediated delivery of wild-type *MFRP*. Furthermore, this study provided novel insights into the role of MFRP in RPE physiology, including (1) modulating actin polymerization and (2) an antagonistic dose-dependent relationship between MFRP and CTRP5 proteins.

1.3.3 hiPSC Models of Other RPE-Related Disorders

Several groups have demonstrated the role of utilizing iPSC-RPE cells to model pathophysiological events in other retinal degenerative disorders including Best Vitelliform Macular dystrophy (BVMD) [69, 135], Gyrate Atrophy [127], and Leber Congenital Amaurosis (LCA) [162].

Given the lack of animal models that recapitulate the BD pathology [105, 106], exclusive localization of BEST1 in a single-cell type, RPE, in the retina [163] involvement of numerous *BEST1* mutations (>200) [62, 164] in the disease, and phenotypic variability between the several different classified bestrophinopathies (AVMD [61], ARB [62], ADVIRC [63], RP50 [64]), hiPSC-based disease modeling and molecular studies are particularly well-suited for studying BEST1 function and the consequence of specific disease-causing mutations on BEST1/RPE cell function in the disease. Moshfegh et al. generated patient-specific hiPSC-RPE from three

different patients harboring the mutations R218H, A243T and L234P in the *BEST1* gene and utilized a novel biosensor imaging system to demonstrate impaired Cl^- ion efflux in patient-derived hiPSC-RPE compared to control hiPSC-RPE thus suggesting a putative role of BEST1 in regulation of Cl^- ions across RPE cell membrane [66]. Li et al. utilized electrophysiological studies on hiPSC-RPE from patients with two distinct *BEST1* mutations, P274R and I201T, to show defective Ca^{2+} dependent Cl^- currents in mutant BEST1 hiPSC-RPE cells [65]. Remarkably, this defect was rescued by viral supplementation of wild-type *BEST1*. Marmorstein et al. used hiPSC disease modeling approach to investigate the pathogenesis of ARB [68]. Their specific focus was on the recessive inheritance pattern of ARB that is postulated to be the result of nonsense mediated decay (NMD) that represents null phenotype for *BEST1* [62]. Utilizing hiPSC-RPE from ARB patients harboring compound heterozygous *BEST1* mutations and unaffected controls, they demonstrated that patient-derived hiPSC-RPE display detectable levels of BEST1 mRNA but reduced levels of mutant *BEST1* compared to control hiPSC-RPE cells. Furthermore, consistent with the disease pathology, ARB hiPSC-RPE in this study also showed impairment of POS internalization and phagocytosis. Highlighting a role of defective POS handling in bestrophinopathies, Singh et al. had also previously utilized hiPSC-based disease modeling on two patients harboring distinct mutations in *BEST1* (A146K and N296H) and showed defects in POS degradation by BD hiPSC-RPE compared to control hiPSC-RPE [69]. Overall, these studies have provided insights into both the function of BEST1 in human RPE cells as well as the pathophysiology underlying bestrophinopathies.

Gyrate Atrophy is a progressive autosomal recessive disorder with childhood-onset inducing diffused atrophy of the choroid, RPE and sensory retina caused by mutations in *OAT1*. Meyer et al. utilized hiPSCs to generate patient-derived optic-vesicle like structures and RPE. Importantly they showed that disease-specific functional defect, reduced OAT activity, could be targeted by both gene repair and Vitamin B6 supplementation of gyrate atrophy-hiPSC-RPE cultures [127].

LCA is a rare autosomal recessive retinal disorder associated with early onset of visual loss, pigmentary and retinal abnormalities, nystagmus and reduced electroretinogram responses. Mutations in at least 20 different genes, including *RPE65*, have been identified as causative in LCA [165]. Using a combination of genome sequencing and a hiPSC-based approach, Tucker et al. identified a novel intronic *RPE65* mutation, IVS3-11 A>G*RPE*65 in LCA. Notably, using an hiPSC-approach the authors demonstrated that the pathogenicity of this novel intronic mutation (IVS3-11 A>G*RPE*65) causes induction of abnormal splicing, translational frameshift and insertion of a premature stop codon [162].

A recent study by Chichagova et al. utilized a hiPSC-based approach to generate hiPSC-RPE from patients with m.3243A>G mitochondrial DNA mutation that is implicated to manifest a range of neurological and ocular phenotypic features [166]. In relation to ocular disease manifestation, patients harboring the m.3243A>G mutation exhibit progressive vision loss with retinal and macular dystrophy [167–169]. The authors demonstrated RPE dysfunction including inability of patient hiPSC-RPE to efficiently phagocytose POS, correlating with lipofuscin accumulation in

postmortem eyes of patients [166, 170, 171]. Additionally, m.3243A>G mutation manifested ultrastructural aberrations of mitochondria, hollowed melanosomes, and decline in apical microvilli abundance in patient hiPSC-RPE cells.

1.4 Innovation, Limitations and Plausible Future Approaches for hiPSC-Based Disease Modeling Strategies Focused on Retinal Degenerative Diseases

The brief synopsis of the hiPSC/hESC studies for interrogating retinal degenerative diseases (Sect. 1.3) shows that this human cell model platform has already been successfully utilized to gain important insights into the pathomechanism of both early onset (e.g., Best disease [65, 66, 68, 69]) and late-onset (e.g., AMD [22, 136, 137, 144, 148, 172]) retinal diseases. Remarkably, the utility of hiPSC-derived target cells as a platform to investigate and therapeutically target RDDs has incorporated a variety of different approaches, including gene therapy and drug screening/testing (Fig. 1.1a). Furthermore, the utility of standalone photoreceptor and RPE cultures (derived from patient's own cells) and when relevant, non-diseased physiological stressor (e.g., POS, serum, A2E) has provided a unique strategy to dissect the singular effect of a specific cell type (photoreceptor vs. RPE), in the absence of complex RPE-photoreceptor interaction, on disease-specific molecular and pathological changes (Fig. 1.1b). For instance, by utilizing patient-derived hiPSC-RPE cells from patients with AMD [136, 137, 144, 148, 172] and related macular dystrophies (SFD, DHRD [22]), several groups have recently shown that cellular defects localized to RPE cells are singularly sufficient to cause both molecular (e.g., alterations in complement pathway genes [22, 136, 137]) and pathological structural alterations (e.g., formation of drusen-like basal deposits [22, 137]) in these diseases. Importantly, these studies have provided a cell culture platform where a precise molecular defect in a specific retinal cell type can be directly linked to disease-characteristic clinical phenotype (e.g., autofluorescence accumulation, drusen formation) in a patient-derived human model of the disease. This is particularly relevant given the fact that numerous RDDs affecting the outer retina, impact both the RPE and photoreceptor cell layer. Furthermore, because the photoreceptor-RPE layer in the retina acts as a functional unit, determining the consequence of cell-specific defects in the disease development and progression in vivo has proven difficult. Of note, the capability to mimic pathological phenotype(s) like autofluorescence accumulation, that is a result of chronic physiological insults and develop over time has been assisted by the fact that hiPSC-derived target cells like RPE and photoreceptors (unlike previous cell culture models) can be cultured for an extended period of time (>3 months) [22, 173–176].

Although major advances have already been made utilizing hiPSC-based disease modeling of specific RDDs, it is important to realize that there are limitations of both using a cell culture model derived from hiPSC/hESCs and current disease

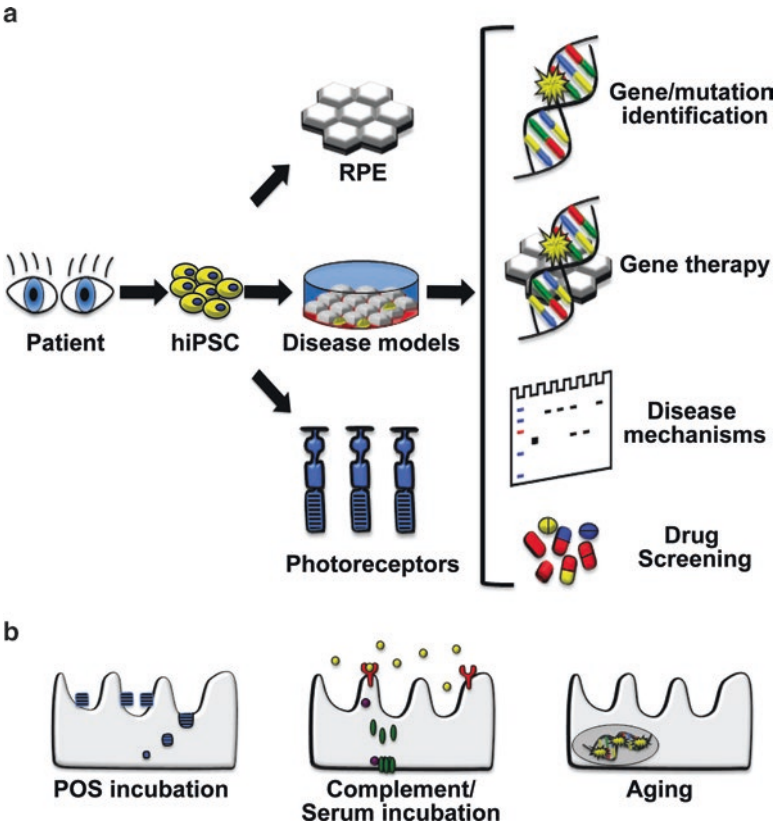

Fig. 1.1 The current hiPSC-based in vitro approaches for retinal degenerative diseases. (**a**) Schematic showing the differentiation of patient-derived hiPSCs to photoreceptor and RPE cells to create a human cell model of the disease that is subsequently utilized for multiple applications including gene/mutation identification in the disease, gene therapy, disease mechanism studies, and drug screening/identification. (**b**) Physiological stressors utilized to metabolically stress hiPSC-RPE in culture that includes exposure to POS, complement/serum, and aging the cells with prolonged time in culture

modeling approaches utilizing a single-cell layer (e.g., photoreceptor, RPE cells) for molecular and therapeutic (gene therapy, drug screening) studies. For instance, hiPSC-RPE in culture lacks the complexity of functional and structural interactions with other cell type(s), including photoreceptors in the retina. Furthermore, hiPSC generation resets their developmental clock, and therefore, hiPSC-RPE in culture are relatively young, and this can pose challenges for studying late-onset diseases, like AMD [177]. As mentioned previously, to overcome these issues, several approaches have been utilized. For example, pharmacological and physiological stressors have been used to metabolically stress and mimic aging of RPE cells in culture [69, 166, 178]. However, there are legitimate concerns with the use of hiPSC/hESC-derived cells that need to be considered in each individual study.

The biggest concern with a cell culture-based model is that of variability, and there are several different reasons underlying the variability in a patient-derived hiPSC model. For instance, a study incorporating multiple patient samples to model and study a disease in an hiPSC-based model system could result in a different cellular phenotype of the same disease in an hiPSC-based model due to variability in the genetic background and clinical presentation of distinct patients. This is consistent with studies in numerous RDDs, where patients harboring the same gene mutation present with different clinical symptoms and pathological characteristics [179]. Another confounding factor is clonal variability, specifically different hiPSC clones generated from the same individual having drastically different cellular characteristics [180]. Furthermore, even in studies limited to a single hiPSC clone per patient sample (a single clone), serial passaging could lead to several changes in the cellular characteristics, including introduction of undesired mutations and chromosomal abnormalities [181]. The fidelity of differentiation is a unique variable in hiPSC-derived cell populations, which becomes relevant when hiPSCs are used to produce two or more cell types that are involved in the disease process, where the percentage of cells forming one cell type vs. other could vary significantly between two distinct hiPSC differentiation runs. Ultimately, there is also the possibility of differences arising between different wells of the same differentiation or differences between cell types in the same well. The resolution of many of these issues is careful study design, increased sample size of experiments, cell population purification (to establish consistency of cell culture for use in downstream experimentation), and establishment of strict quality control metrics. For instance, to account for differences in genetic background and clinical presentation, when the possibility of variable phenotypes has previously been demonstrated in clinical studies, a plausible disease modeling approach would be to group patient samples by phenotype with the inclusion of isogenic control/gene-corrected line (in inherited diseases with known genetic defect) for each patient line. Another approach to resolve this situation would be to use hiPSC-based disease modeling to study diseases that are solely by a singular gene defect with complete penetrance. To account for clonal variability, each individual clone should be thoroughly characterized before experimentation. Furthermore, usage of nonintegrating plasmid vectors for reprogramming and karyotyping of all clones can ensure the absence of any unwanted genetic mutation and chromosomal rearrangement. Genome sequencing can also be used to verify that clones from the same individual are genetically consistent. Most importantly to overcome the variability and likelihood of false-positive results, before formulating any conclusions about the molecular/cellular changes between control and patient cells, it would be important to ascertain that the results are consistent after analysis of multiple clones of patient and control hiPSCs. Similarly, limiting the passage number of hiPSCs utilized in the study helps to maintain genomic integrity that can be monitored through genome sequencing and karyotypic analyses at different passages eliminating variability arising from serial passaging in hiPSC-derived cultures. Ultimately, the baseline characterization of the target cell type (e.g., RPE) in each differentiation run, utilizing some defined criteria (e.g., morphology, pigmentation, polarity) is critical for meaningful experimentation and data interpretation in an hiPSC-derived model system.

The various strategies that have been used for hiPSC-based disease modeling include utilizing isolated cell type(s), complex cell models, 3D culture system, and human–animal chimeras [174, 182, 183]. Of note, as shown by the various examples cited here (see Sect. 1.3), some of these approaches have already been utilized for study of RDDs using hiPSCs. The utility of a single approach is dependent on what phenotypically mimics the disease most accurately with limited complexity. Individual cell types are most relevant when the disease is caused due to dysfunction of a singular cell type, and in case of a genetic disease, the gene responsible for the disease is expressed by that cell type. Alternatively and more complex, interaction between two or more cell type(s) or a specific tissue in its entirety is required for studying the disease pathogenesis and disease pathology. To address such a scenario, complex cell models incorporating multiple cell layers (e.g., photoreceptor-RPE-choriocapillaris) may be necessary (Fig. 1.2). This would first necessitate bioengineering individual cell layers that physiologically and functionally recapitulate their in vivo counterpart (photoreceptor, RPE, choriocapillaris). Important in the proposed scenario, significant advances have already been made into address this goal in both photoreceptors and the RPE [154, 183]. 3D culture systems are also uniquely suited for studying diseases where the complex microenvironment surrounding the cells in vivo is relevant for disease development. 3D culture systems can achieve compartmentalization of different cell type(s) or help promote cellular polarity in an in vitro model system. Finally, the most complex strategy is the generation of humanized animal model. Both hiPSC and hiPSC-differentiated

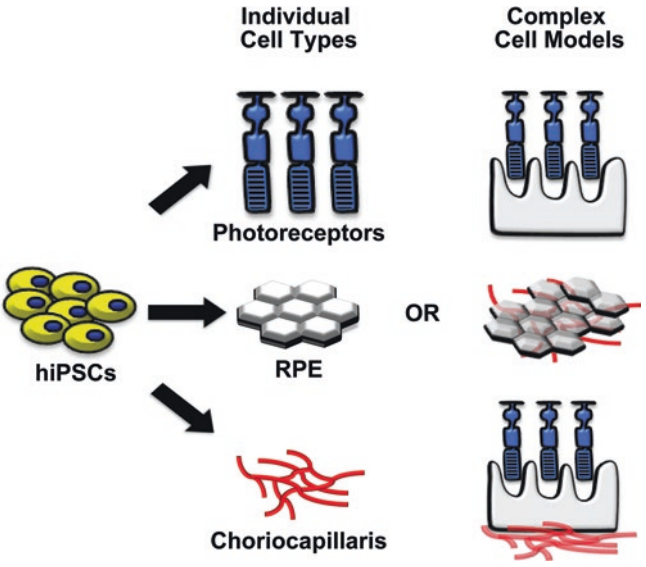

Fig. 1.2 The incorporation of individual cell layers vs. complex cell models in hiPSC-based disease modeling of retinal diseases. Schematic representing the current and future approaches to the utility of a single-cell layer (RPE vs. photoreceptor vs. choriocapillaris) and complex retinal cell models (photoreceptor-RPE, RPE-choriocapillaris, and photoreceptor-RPE-choriocapillaris) in retinal degenerative disease modeling and therapeutic studies (gene therapy, drug screening)

target cells have been injected into animal models (brain, retina), and preliminary studies have shown that these cells can integrate into the mouse tissue and yield humanized neurons and glia [184–186]. However, their utility in modeling and studying RDDs has not yet been established.

1.5 Conclusion

The use of pluripotent stem cell technology has revolutionized our approach to study and therapeutically targeting human diseases and has put the concept of personalized medicine within grasp. This is particularly relevant to RDDs that are a major cause of blindness in children and adults and where few therapies currently exist to target these debilitating disorders. Importantly, over the last decade, we have identified several hundreds of genes that are responsible for specific RDDs (266, https://sph.uth.edu/retnet/sum-dis.htm#B-diseases), but the disease mechanisms in most of these diseases still remain unresolved. Future studies in a patient-derived hiPSC model system are bound to increase our understanding of the molecular basis of several of these diseases thereby leading to the development of rational drug therapies.

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