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Concise Review: Making Stem Cells Retinal: Methods for Deriving Retinal Pigment Epithelium and Implications for Patients With Ocular Disease

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Key Words. Retinal pigment epithelium • Embryonic stem cells • Induced pluripotent stem cells • Retinal diseases • Macular degeneration • Cell differentiation

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ABSTRACT

Stem cells provide a potentially unlimited source of cells for treating a plethora of human diseases. Regenerative therapies for retinal degenerative diseases are at the forefront of translation to the clinic, with stem cell-derived retinal pigment epithelium (RPE)-based treatments for age-related macular degeneration (AMD) already showing promise in human patients. Despite our expanding knowledge of stem cell biology, methods for deriving cells, including RPE have remained inefficient. Thus, there has been a push in recent years to develop more directed approaches to deriving cells for therapy. In this concise review, we summarize recent efforts that have been successful in improving RPE derivation efficiency by directing differentiation from human pluripotent stem cells using developmental cues important for normal RPE specification and maturation in vivo. In addition, potential obstacles for clinical translation are discussed. Finally, we review how derivation of RPE from human induced pluripotent stem cells (hiPSCs) provides in vitro models for studying mechanisms of retinal disease and discovering new avenues for treatment. STEM CELLS *2015;33:2363–2373*

INTRODUCTION

The advent of human embryonic stem cells (hESC) in 1998 [1] and human-induced pluripotent stem cells (hiPSC) less than a decade later [2] has provided potentially unlimited supplies of source material for cell-based therapies (hESC and hiPSC), and an invaluable tool for studying human retinal degenerative diseases in vitro (hiPSC). There are many advantages to developing regenerative treatments for use in the eye, including the small size relative to other organs (fewer cells would be sufficient for therapy), ease of accessibility, potential immune privilege, and separation from systemic circulation by the blood-retinal barrier. Additionally, there are well-established methods for early disease diagnosis, for monitoring improvements (or deficits) in a patient's vision after treatment, and for observing transplants noninvasively once administered into the eye. Diseases of the eye present a large healthcare burden and greatly impact the quality of life of afflicted individuals. Thus, efforts to translate treatment to the clinic are in full force and many groups are specifically working towards a cure for age-related macular degeneration (AMD), which is the leading cause of blindness in people over the age of 65 years in the developed world [3, 4].

AMD is a progressive disease caused by death and dysfunction of the retinal pigment epithelium (RPE) in the region of the retina called the macula, which is critical for detailed central vision. The RPE carries out a number of functions essential for perpetuating the visual cycle and maintaining the health and function of the photoreceptors (RPE function reviewed in ref. [5]); thus, when the RPE dies, the photoreceptors die and vision is lost. Early AMD can progress into two forms and, at present, there are few therapeutic options patients with advancing disease can seek for treatment. In some cases, intraocular injections of vascular endothelial growth factor (VEGF) inhibitors [6], such as Avastin, Lucentis, and Eylea, can quell abnormal blood vessel growth typical of the "wet," or exudative, form of AMD; however, there is no current treatment option for the more common "dry," or atrophic, form, where RPE and photoreceptors are lost in diseased regions of the retina (AMD reviewed in ref. [6]). Because loss of a specific cell type leads to disease progression, AMD is a candidate disease for developing cell-based therapies. Given the etiology of AMD and the fact that the "dry" form constitutes 80%–90% of patient cases [7], there has been a massive effort in recent years to differentiate RPE from

human pluripotent stem cells (hPSC) for use in transplantation studies. Many of these studies have used the Royal College of Surgeons (RCS) rat as a model of retinal dystrophy and have shown visual improvements in these animals after transplantation with hPSC-derived RPE [8–11]. More recently, a phase 1/2 clinical trial administering a bolus injection of hESC-derived RPE into the subretinal space of atrophic AMD and Stargardt's disease (juvenile onset macular degeneration) patients has demonstrated safety [12, 13]. In Japan, monolayers of hiPSC-RPE have been transplanted into patients with exudative AMD [14, 15].

In this review, methods for generating RPE from hPSC will be discussed focusing on newly developed directed differentiation protocols, which improve efficiency of RPE derivation. A brief summary of two differentiation protocols used to generate RPE for clinical AMD studies will be included. In addition, anticipated obstacles for translating these directed protocols to meet clinical standards defined by the U.S. Food and Drug Administration (FDA) will be considered. Finally, recent reports using patient-derived hiPSC to model human retinal diseases will be reviewed, highlighting the benefits of using directed differentiation methods for future investigation in this field.

Directing Differentiation of Human Pluripotent Stem Cells to RPE

It has been well demonstrated that hPSC have the capacity to "spontaneously" differentiate to RPE (among other cell types) using the continuous adherent culture method [8, 9, 16–19] and the embryoid body method [10, 17] and both methods are currently used to generate RPE for AMD clinical studies (these methods are reviewed in ref. [20, 21]). The continuous adherent culture method allows stem cells to overgrow in the absence of basic fibroblast growth factor (bFGF) for an extended period of time (up to 14 weeks), during which pigmented foci begin to emerge and expand, eventually growing big enough to manually dissect out of the culture dish for enrichment [20]. Generally, it takes between 1 and 8 weeks from removal of bFGF for pigmented foci to become visible [8, 16–19]. In contrast, the embryoid body method involves suspension culture of neurospheres for 1–3 weeks with subsequent seeding onto an adherent substrate [20]. Initial pigmentation is seen between weeks 4 and 8 using this method [11, 22, 23]. While both approaches yield highly pure cultures of RPE, they are lengthy and inefficient. Efforts to improve these limitations have focused on directing RPE differentiation by supplementing media with growth factors and small molecules that mimic exogenous signals known to be important for RPE development in vivo (RPE development is reviewed in ref. [24]).

A number of recent reviews have outlined existing protocols for directing RPE differentiation from hPSC [21, 25–27] and a summary of published directed differentiation methods can be found in Table 1. Several protocols from 2008 to 2009 [28–30] use a serum-free floating culture of embryoid body-like aggregates (SFEB) system, which has been shown to favor generation of neural precursors over mesodermal or endodermal lineages [31]. Neural induction can be enhanced in this system by inhibiting endogenous Wnt and Nodal signals using Dkk1 and Lefty A (SFEB/DL), respectively [31–33]. Earlier directed differentiation methods determined the ability of hPSC to differentiate to RPE by replating SFEB/DL-treated aggregates grown in suspension (for 20 days) onto plates with poly-D-lysine–laminin–fibronectin-coated substrates [28–30]. Substrates such as type I collagen [34], laminin [11], Matrigel [35, 36], and fibronectin [37] have also been used in RPE differentiation studies from hPSC. Using the SFEB/DL approach, close to a third of generated cells showed visible pigment (approximately 37% [29]) or were positive for markers of RPE progenitors, coexpressing MITF and PAX6 (approximately 30% [30]). Modifications to this protocol have been made, resulting in successful derivation of RPE as well [22]. SFEB differentiation media could be supplemented with small-molecule alternatives to Dkk1 and Lefty A (CKI-7 and SB431542, respectively) and similar results were achieved [28]. Small molecules are often preferred to recombinant proteins because of their low cost and clinical translatability and many groups have taken this approach when developing or modifying protocols. Lane et al. [38], for example, showed that adding the small-molecule neuralizing factor, dorsomorphin, to the continuous adherent culture method increased pigmented foci during spontaneous differentiation to RPE.

Other directed methods have tested the effects of nicotinamide (NIC), which plays a role in cell proliferation and differentiation [39], on deriving RPE from hPSC. When free-floating clusters were treated with NIC for 4 weeks, RPE-specific genes were upregulated and pigmented patches were visible in differentiating hESC aggregates [11, 35]. This effect was augmented when Activin A, a member of the TGF-β superfamily and known promoter of the RPE lineage [40], was added to the media alongside NIC later on in the differentiation protocol (weeks 3–4) [11, 35, 41]. Although these protocols used a more directed approach than the SFEB system (using known RPE-promoting signals, like Activin A), efficiencies were still low, with only 30%–40% of cells positive for pigment [11, 41]. Importantly, these studies more rigorously tested the functionality of derived RPE in vitro and in vivo, whereas previous reports had only examined phagocytic ability of the cells in a culture dish. Results from in vitro assays confirmed phagocytic ability and demonstrated polarized secretion of biomolecules [35, 41]. Additionally, derived RPE had membrane potential and Na^+ and K^+ ion transport similar to native cells [41]. In vivo, derived RPE brought about survival of photoreceptors in the dystrophic RCS rat [11, 35].

More recently published protocols have tried to recapitulate what is known about early eye and RPE development to more effectively direct differentiation to RPE in a stepwise manner [42–46]. A study by Zahabi et al. [42] examined the effects of sequentially adding neural differentiation factors (noggin [47], retinoic acid [48], SB431542 [28]), the neural retina inducing FGF signal [49], and RPE-promoting sonic hedgehog (Shh) [50] signals to media over 18 days of differentiation. With extended culture postdifferentiation, this protocol yielded >50% of cells positive for MITF and approximately 40% of cells positive for the mature RPE marker, RPE65, on day 60, although function of these cells was never determined [42]. Buchholz et al. [43] developed a protocol using factors previously shown to induce neural retinal progenitors from hESC with high efficiency (noggin, insulin-like growth factor [IGF1], Dkk1, and bFGF) [51] and known RPE-

Table 1. Methods for directing differentiation of human pluripotent stem cells (hPSC) to RPE

References	Culture type		Efficiency		Functional assays
	hPSC type	Media supplementation	%	Quantification method	
	Suspension culture				
Osakada et al., 2008 [29] Hirami et al., 2009 [30] Osakada et al., 2009 [28]	hESC [28, 29] hiPSC [28, 30]	Lefty A and Dkk1 (20 days) [29, 30] or CKI-7 and SB431542 (21 days) [28] *Transitioned to adherent culture*	*hESC* 32.5 ± 3.0 [28] 37.4 ± 4.6 [29] *hiPSC* 29.0 ± 3.3 [28] 29.8 ± 4.0 [30]	Pigmented cells (day 60) [29] MITF+/PAX6+ cells (day 35) [30] MITF+ colonies (day 30) [28]	Phagocytosis [28, 29]
Idelson et al., 2009 [11] Krohne et al., 2012 [35]	hESC [11] hiPSC [35]	NIC (1 week) *Transitioned to adherent culture* NIC, activin A (weeks 3–4)	33.4 ± 3.8 [11]	Pigmented cells (6 weeks)	Transplantation into RCS rat (rhodopsin+ in vivo [11] and rescue of photoreceptors [35]), phagocytosis and PEDF/VEGF secretion [35]
Kokkinaki et al., 2011 [41]	hiPSC	NIC, activin A (week 3–4) *Transitioned to adherent culture* NIC (3–5 weeks)	37 ± 6.5	Pigmented clusters (8–9 weeks)	Phagocytosis, basal VEGF secretion, electrophysiology
	Adherent culture				
Zahabi et al., 2012 [42]	hiPSC	Noggin or SB431542, bFGF (day 0–6); all-trans-retinoic acid, bFGF (day 6–12); Shh, bFGF (day 12–18)	57.4 ± 8.1 and 39.4 ± 4.5	MITF+ and RPE65+ cells (day 60)	N/A
Buchholz et al., 2013 [43] Leach et al., 2015 [45]	hESC [43, 45] hiPSC [43]	Noggin, IGF1, Dkk1, NIC (day 0–2); supplemented with bFGF (day 2–4); IGF1, Dkk1, activin A (day 4–6); activin A, VIP, SU5402 (day 6–14) [43] or CHIR99021, activin A, SU5402 (day 8–14) [45]	*hESC* 78.5 ± 1.2 and 79.8 ± 0.88 [43] 97.77 ± 0.1 [45] *hiPSC* 63 ± 0.88 [43]	PMEL17+ cells (day 14)	Phagocytosis [43, 45], PEDF/VEGF secretion [45]
Maruotti et al., 2013 [61]	hESC, hiPSC	Differentiation medium (day 0–50), then RPE medium (+B27, day 50–115) with serial passaging	Day 50 16.6 ± 2.5 (hESC) 16.1 ± 0.1 (hiPSC) Day 115 97.6–98.5	RPE65+ cells	Phagocytosis, basal VEGF secretion, transplantation into albino NOD-SCID mice (rhodopsin+ *in vivo*)
Lane et al., 2014 [38]	hESC (only Shef3 line)	Spontaneous differentiation method modified with dorsomorphin (day 0–10)	17.7 ± 3.3 (foci/cm^2)	Pigmented foci	N/A

Table 1. *Continued*

References	Culture type		Efficiency		Functional assays
	hPSC type	Media supplementation	%	Quantification method	
3D aggregates and retinal structures					
Meyer et al., 2009 [56] Phillips et al., 2012 [57] Singh et al., 2013 [58] Zhong et al., 2014 [59]	hESC [56] hiPSC [56–59]	NIM (N2, heparin) *Transitioned to adherent culture* NIM and RDM (day 16 +B27)	*hESC* 25% [56] *hiPSC* 19.0 ± 7.2 [59]	MITF+ cells (day 40 [56] or between days 21–28 [59])	Phagocytosis, PEDF/VEGF secretion, barrier function (TER), Ca^{2+} response [58]
Zhu et al., 2013 [44] Nakano et al., 2012 [46] Reichman et al., 2014 [63]	hESC [44, 46] hiPSC [44, 63]	Embedded in Matrigel, NIM (N2B27, until day 5) [44] Matrigel, IWR1, FBS, SAG (MIFS, day 0–18); CHIR99021 (day 15–18) [46] NIM (N2) [63] *Transitioned to adherent culture* Activin A (day 6–30) [44] NIM (N2, day 14–30) [63]	*hESC* 95.7 ± 1.0 [44] *hiPSC* 48.0 ± 6.2 [44]	Pigmented cells (day 30) [44]	Phagocytosis [44, 63], barrier function (TER) and transplan- tation into RSC rat (photore- ceptor rescue) [44]

Abbreviations: bFGF, basic fibroblast growth factor; FBS, fetal bovine serum; hESC, human embryonic stem cells; hiPSC, human induced pluripotent stem cells; IGF1, insulin-like growth factor 1; NIC, nicotinamide; NIM, neural induction medium; PEDF, pigment epithelium-derived factor; RDM, retinal differentiation medium; RSC rat, Royal College of Surgeons rat; SAG, smoothened (hedgehog) agonist; Shh, sonic hedgehog; TER, transepithelial resistance; VEGF, vascular endothelial growth factor; VIP, vasoactive intestinal peptide.

promoting factors (NIC, Activin A, vasoactive intestinal peptide [VIP], and SU5402). Adding combinations of these factors to differentiation medium over the course of only 14 days resulted in high efficiency generation of cells with an RPE phenotype. Based on PMEL17 expression, the 14-day protocol yields approximately 80% RPE from human stem cells of embryonic origin and approximately 63% RPE from hiPSC [43]. Improvements in RPE derivation using the 14-day method have been made by modifying hESC passaging techniques (approximately 85% efficiency based on PMEL17 expression [45]) and by modulating other known RPE-promoting cues, like the Wnt signaling pathway [46, 52–54]. Using fluorescent reporter hESC lines, our group has found that adding a small molecule agonist of Wnt signaling, CHIR99021, to the 14-day differentiation protocol significantly improved RPE derivation [45]. Furthermore, 14-day-derived cells retain RPE identity after multiple passages using expansion and preservation methods adapted from protocols currently generating RPE of clinical quality [12, 45, 55] (Fig. 1).

Differentiation methods that recapitulate optic patterning events in vitro, yielding three dimensional (3D) retinal structures capable of forming both neural retina and RPE tissues, have also been developed. Several studies have cultured hPSCs as suspension aggregates in neural induction media containing N2 and heparin, then switched to an adherent culture in retinal differentiation media containing B27 [56–59]. This method yields approximately 20% RPE based on MITF expression [56, 59] and cells excised from these differentiated cultures and expanded were shown to carry out RPE functions in vitro [58]. A method deriving RPE from 3D spherical neural masses (SNMs) has proven successful as well, with approximately 5% of vesicular structures yielding functional RPE [60]. Although reported RPE yields were low, a common advantage of these studies is that differentiation was directed using media supplementation and not exogenous factors, and this may be more amenable to scaling up production. A similarly simplistic approach has been taken using adherent culture, where media supplemented with B27, combined with serial passaging of cells yields >97% RPE by day 115 [61].

In 2012, Nakano et al. reported that self-forming 3D optic cup-like structures could be generated using stepwise addition of different lineage promoting factors [46]. Using SFEB culture, neuralization of hESC was induced by adding extracellular matrix (Matrigel) and inhibiting Wnt using the small molecule antagonist, IWR1. Retinal differentiation was enhanced over 18 days with the combined effects of IWR1, fetal bovine serum (FBS), and Shh signaling activation. Formation of MITF-positive epithelium was achieved by adding CHIR99021, from days 15 to 18 of differentiation, and by days 19–24, a portion of aggregates invaginated forming layered optic cups where the outermost tissue expressed MITF (RPE) and the innermost tissue expressed CHX10 (neural retina) [46]. The neural retinal tissue of these 3D optic cups formed stratified cell layers that expressed markers for ganglion cells, photoreceptors, and other retinal progenitors. This method was recently modified to include transient treatment with BMP4 in place of Matrigel early in the protocol, which improved retinal differentiation [62]. Other reports of self-forming 3D retinal structures used a simpler approach by culturing cells in media supplemented with N2 [63] or combining Matrigel encapsulation of hPSCs with N2/B27-based induction medium followed by directed

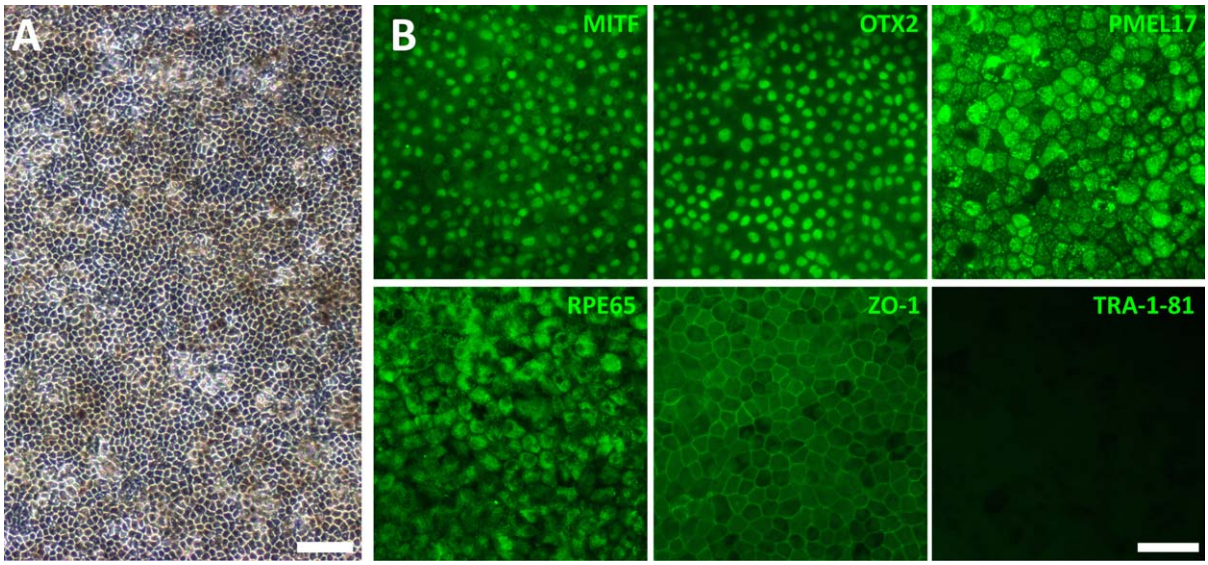

Figure 1. Culture day 30 passage 3 (p3) human embryonic stem cell (hESC)-retinal pigment epithelium (RPE) derived by directed differentiation pigment and express RPE markers. RPE were derived using a previously published 14-day directed differentiation protocol [43]. **(A):** Phase contrast image showing p3 hESC-RPE pigment by culture day 30. Scale bar = 100 μm. **(B):** Fluorescent images show day 30 p3 hESC-RPE cultures label positive for MITF, OTX2 (RPE markers), PMEL17 (pigmentation marker), RPE65 (visual cycle marker), and ZO-1 (tight junction marker). Importantly, p3 hESC-RPE does not express TRA-1–81, which is a marker of pluripotent cells. Scale bar = 50 μm.

differentiation to RPE with Activin A [44]. This latter approach by Zhu et al. [44] was highly efficient in producing RPE and the details of this approach are summarized in the following section.

It is worth pointing out that studies generating RPE from hiPSC alongside hESC show reduced efficiency from hiPSC and differences range from being minor [28] to considerable [43, 44] depending on the method of directed differentiation. Moving forward, it may be important to define factors that specifically enhance RPE derivation from hiPSC. The findings outlined in this section demonstrate the potential directed differentiation protocols hold for speeding clinical application of RPE therapies (and possibly combined RPE-neural retina therapies) for treating AMD and other retinal degenerative diseases. As these protocols have been devised to mimic known developmental cues, directed differentiation methods also provide a unique model in which to study human RPE differentiation in a shorter time frame relative to in vivo ocular development.

Considerations for Clinical Translation of Directed Differentiation–Derived RPE and Potential Obstacles to Progress

RPE-based therapies generated using directed differentiation methods face the same challenges of any allogeneic cell-based therapy being developed for the clinic. HiPSC offer a potential source of autologous cells for therapy, but given the cost limitations and length of current RPE derivation protocols, using human leukocyte antigen-matched donor hiPSC-RPE might be a more feasible option. There are currently two clinical studies underway testing the safety and efficacy of stem cell-based therapies in human patients with macular degeneration: one using bolus injection of hESC-derived RPE [12, 13] and another using transplanted hiPSC-derived RPE sheets without a scaffold [14, 15]. An unanswered question remains as to what is the best method to deliver RPE to the back of the eye? Suspensions of hPSC-derived RPE injected into the subretinal space of the dystrophic RCS rat can preserve visual function [8–10], however placement and survivability of cells can be problematic [9, 55]. Transplantations of hPSC-derived RPE as a monolayer sheet both with and without a supporting scaffold have been developed as an approach to surmount these limitations, and RPE sheets without a scaffold have shown improvements in photoreceptor survival and function in animals [34]. A study comparing methods of RPE delivery showed significant improvements in cell survival and presence of a preserved RPE monolayer in animals receiving cells on a scaffold compared with cells in suspension [55]. Recently published results from the hESC-RPE clinical trial show that bolus transplants were well tolerated by patients with AMD and Stargardt's macular dystrophy, that no teratomas formed during the time patients were monitored, and that more than half the eyes treated were reported to show improvement in visual acuity [13]. As clinical trials using both transplantation methods are in early phases, comparability in humans remains to be established.

The RPE generated for these clinical studies were made under conditions adapted to meet the FDA's current Good Manufacturing Practices (cGMP) and minimal manipulation standards [64, 65]. These guidelines generally recommend that cell-based therapeutics are derived in the absence of xenogeneic or adventitious agents (termed xeno-free) and are minimally altered during processing before use for therapy. It has been shown by several groups, including those proceeding with clinical trials, that RPE can be derived from hPSCs under xeno-free conditions using the "spontaneous" approach to RPE differentiation [10, 12, 13, 66–68]. As mentioned previously, however, this approach is time consuming, resource consuming, and inefficient, which represents a bottleneck for translation to the clinic. More efficient methods are being sought; however, there are no published RPE-directed

differentiation protocols that meet cGMP requirements. Replacing recombinant proteins with small molecules or cGMP-grade analogs when developing new protocols or modifying existing ones will be an important step moving forward.

Excessive manipulation of RPE during manufacturing can result in cell senescence and epithelial to mesenchymal transition [69], which, in addition to cGMP compliance, is of concern for the FDA. In 2013, Zhu et al. published a directed RPE differentiation method where hESC were embedded in Matrigel in neural induction medium and allowed to differentiate as floating neuroepithelial cysts [44] (Table 1). After 5 days, cells were transitioned to adherent culture conditions in the presence of Activin A until day 30, when approximately 95% of cells derived from hESC were determined to be pigmented [44]. While the reagents used for this protocol were not cGMP-compliant, they found that day 30 RPE did not require manual enrichment or selection for expansion, unlike several directed methods described in the previous section [11, 41–43]. In fact, this study showed cells at day 30 were functional in vitro and could rescue photoreceptor degeneration when transplanted into the dystrophic RCS rat without prior manual selection [44]. Further improving the efficiency and purity of RPE derivation using protocols compliant with cGMP standards will decrease the amount of processing and handling of cells. This will be a critical step towards getting speedier methods of generating RPE for patient use to the clinic.

As directed RPE differentiation methods improve, researchers who have also established successful hPSC-RPE-based therapies derived by the "spontaneous" method may encounter the unique obstacle of demonstrating comparability to the original cells, which may involve repeating preclinical or even early clinical studies. When changes are made to aspects of the manufacturing process, comparability before and after process modification needs to be established using in vitro (and, if necessary, in vivo) assays [70]. For example, a recent study of mesenchymal stem cells showed considerable variability in critical attributes, despite having been isolated from the same source [71].

The FDA recognizes the need for a case-by-case approach when developing regenerative medicine products and has provided guidelines on preclinical assessment for these types of therapies [72]. Due to the inherent heterogeneity of regenerative medicine products, whether from cell types derived for therapy, manufacturing processes, mechanisms of action, or routes of administration, the earlier preclinical assessments start during product development, the more amenable that product is to clinical investigation [72]. Preclinical testing design should be informed by the complexity of the product as well as framework similarities that can be applied to all regenerative medicine products, such as tumorigenic and toxicity studies [72]. Additionally, regenerative therapies incorporating surgical devices and/or noncellular components (e.g., scaffolds) will likely require additional safety testing regardless of previous approval or testing in humans [72].

Future Approaches and Benefits to Patients: Modeling Retinal Disease in a Dish

While faster and more efficient methods for generating RPE are underway, preclinical studies and clinical trials present a challenging and lengthy path towards therapies. An emerging field of research is focused on differentiating retinal and RPE cells from patient-derived hiPSC with the goal of learning more about the mechanisms of and potential treatments for human ocular disease (reviewed in ref. [27, 73]). This approach, although not without its own drawbacks, will remedy some of the current limitations to studying human retinal degenerative disease using animal models, which may not accurately represent all facets of human disease, or postmortem tissues, where disease may be very advanced making insight into disease progression difficult. The 'disease in a dish' approach to investigating RPE-based disorders can be facilitated by directed differentiation methods, which will accelerate the time it takes to obtain results. Additionally, high RPE yield and efficiency afforded by directed protocols decreases culture heterogeneity and boosts consistency between experiments. For highly complex diseases, where RPE and and/or retinal cell types are affected, such as AMD, modeling disease might be better achieved using stem cell-derived 3D culture systems where stratified tissue structures more precisely mimic the in vivo architecture [56, 57, 60, 73]. This type of approach could use layered RPE and neural retinal tissue derived from optic cups [46], or could coculture RPE with neural retinal progenitors embedded in a matrix [22]. In this section, we will summarize recent studies using patient-derived hiPSC to model ocular disease, specifically focusing on RPE-based studies using RPE derived by both spontaneous and directed methods (Table 2).

A range of diseases have been studied using patient-derived hiPSC-RPE, from retinal dystrophies where RPE and photoreceptor cells are lost as disease progresses (e.g. retinitis pigmentosa) to RPE-related disorders where photoreceptor degeneration is secondary. In 2012, Zahabi et al. showed that RPE could be directed to differentiate from lines of hiPSC derived from patients with different types of common inherited retinal degenerative disorders, including 2 patients with retinitis pigmentosa (RP), and that cells pigmented and expressed markers of true RPE [42]. Retinitis pigmentosa is a genetically diverse form of inherited retinal dystrophy that presents early on in life (anywhere from infancy to middle age) depending on the method of inheritance [74]. Mutations in the *RP2* gene can cause X-linked forms of RP (XLRP) that affect individuals early in this age range [75]. Schwarz et al. derived RPE from XLRP patient-derived hiPSC using the continuous adherent culture method and confirmed the RP2 R120X (Arg120stop) mutation in these cells [76]. Based on the potential role of RP2 protein in vesicle trafficking and cilia function [77], R120X hiPSC-RPE phenotype was further analyzed, showing disruptions in Golgi cohesion and mislocalization of the ciliary marker protein, IFT20, and the transducin G-protein subunit, Gβ1 [76]. Like AMD, there is no cure for RP, and for patients with XLRP, there are no treatments to restore RP2 protein function. Importantly, this study showed that adding translational read-through inducing drugs (TRIDs), G418 and PTC124 (Ataluren), which allow incorporation of an amino acid at the premature stop codon, was able to restore 13%–20% of RP2 protein, correct localization of IFT20 and Gβ1, and resolve Golgi cohesion in R120X hiPSC-RPE [76].

Mutations in the gene coding for an RPE-specific membrane receptor, membrane frizzled-related protein (*MFRP*), has been found to also cause inherited RP. Using MFRP-

Table 2. Summary of studies using patient-derived hiPSC-RPE to model human ocular disease in vitro

Disease(s) modeled	Disease-causing gene (mutation)	Normal function	RPE differentiation method	Summary of important findings	References
RP USH1 LHON LCA	Various	N/A	*Directed* See Table 1 for media composition [42]	*In vitro phenotype* When directed to differentiate, cells pigmented and expressed RPE-associated markers	[42]
RP	*RP2* (R120X)	GTP-ase activating protein (GAP), role in trafficking from Golgi to cilia membrane	*Spontaneous* Continuous adherent culture method	*In vitro phenotype* Golgi dispersion Mislocalization of IFT20 (basal body) and Gβ1 (PM) proteins *Implications for Treatment* TRID (G418 and PTC124) treatment restored 13%–20% RP2 protein, corrected localization of IFT20 and Gβ1, improved Golgi cohesion	[76]
RP	*MFRP*	RPE-specific type II transmembrane protein (function unknown, but this study implicates a role in actin polymerization)	*Directed* NIC, transition to adherent culture in RPE medium (after 6–10 weeks), modified from [11]	*In vitro phenotype* Abnormal morphology and pigment localization Decreased overall pigment and loss of tight junctions Disorganization and increased expression of β-actin protein *Implications for Treatment* AAV-*MFRP* normalized actin filament organization, restored RPE apical microvilli, pigmentation, and epithelial barrier function (tight junctions) Administering AAV-*Mfrp* to mice with mutant Mfrp protein (a preclinical model of RP) rescued photoreceptor survival and function (up to 6 months), showed RPE-specific expression of Mfrp *in vivo*	[78]
CHM	*CHM*	Encodes REP1 (Rab export protein-1) enzyme which enables correct modification (prenylation) and localization (PM) of GTPases (Rabs)	*Spontaneous* Continuous adherent culture method	*In vitro phenotype* Lack of REP1 protein Underprenylation of Rabs Decreased number of membrane-bound Rabs, increased number of cytoplasmic Rabs *Implications for Treatment* AAV2/5-*CHM* normalized Rab prenylation and membrane localization in vitro Successful gene transfer in vivo up to 2 weeks	[80]
Pigment retinopathy (LCHADD)	*HADHA*	Mitochondrial β-oxidation of fatty acids (encodes α-subunit)	*Spontaneous* Embryoid body method	*In vitro phenotype* Decreased pigmentation, smaller size and irregular tight junctions Cytoplasmic accumulation of long chain triglycerides	[84]
BVMD	*BEST1* (A146K and N296H)	Debatable; channel, channel regulator or transporter	*Directed* See Table 1 [56–59] for media composition	*In vitro phenotype* Increased autofluorescent material and oxidative stress, altered calcium homeostasis, decreased fluid flow, delayed RHODOPSIN degradation *Functional Insight* Study links BEST1 to ER-mediated Ca^{2+} release/uptake	[82]

Table 2. *Continued*

Disease(s) modeled	Disease-causing gene (mutation)	Normal function	RPE differentiation method	Summary of important findings	References
GA	*OAT* (A226V)	Vitamin B_6-dependent enzyme, breaks down ornithine	*Directed* See Table 1 [56–59] for media composition; sometimes modified with noggin (or dorsomorphin), Dkk1 (or XAV939), and Activin A	*In vitro phenotype* Impaired OAT enzyme activity *Implications for Treatment* Increased levels of vitamin B_6 (600μM) enhanced OAT enzyme activity	[83]
Microphthalmia	*VSX2/CHX10* (R200Q)	Neural retina-specific transcription factor	*Directed* See Table 1 [56–59] for media composition	*In vitro phenotype* Propensity to differentiate towards RPE (increased pigmentation, decreased expression of neural retina markers, decreased proliferation) Delayed maturation of photoreceptors and absence of bipolar cells VSX2 rescue decreased RPE differentiation	[90]

Abbreviations: AAV, adeno-associated virus; BVMD, Best vitelliform macular dystrophy; CHM, choroideremia; ER, endoplasmic reticulum; GA, gyrate atrophy; GTP, guanosine-5′-triphosphate; LCA, Leber's congenital amaurosis; LCHADD, long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency; LHON, Leber's hereditary optic neuropathy; NIC, nicotinamide; PM, plasma membrane; RP, retinitis pigmentosa; RPE, retinal pigment epithelium; TRID, translational read-through inducing drug; USH, Usher syndrome.

mutant patient-hiPSC to derived RPE, Li et al. [78] showed abnormal morphology, pigment localization, and β-actin distribution as well as decreased overall pigmentation compared with control; additionally, tight junctions were compromised in MFRP-mutant cells. These RPE irregularities could be repaired using an adeno-associated virus (AAV) vector (serotype 2/8) therapy to introduce the correct copy of *MFRP* into patient-derived cells [78]. Furthermore, long-term survival and function of photoreceptors was shown in an established preclinical animal model of RP ($Mfrp^{rd6}/Mfrp^{rd6}$ mice) after AAV-treatment [78]. This approach is instrumental in its potential to show proof of concept for gene therapy in RPE-based disorders (and other diseases) that do not have appropriate animal models. Choroideremia is an inherited pigment retinopathy for which efforts to generate an acceptable animal model has proven difficult and unsuccessful. Choideremia is caused by mutations in the gene, *CHM*, encoding the protein, REP1 (Rab escort protein-1), which functions to correctly modify and localize Rab guanosine triphosphates (GTPases) [79]. Cereso et al. [80] generated hiPSC from a *CHM*-mutant patient, which were differentiated to RPE using the spontaneous continuous adherent culture method. Patient-derived RPE were shown to recapitulate the biochemical defects presenting in choroideremia patients, which were successfully reversed with AAV treatment to introduce a correct version of *CHM* [80]. Importantly, this study compared different AAV vector serotypes, including the serotype (AAV2/2) used to treat patients with Leber congenital amaurosis (LCA) [81] and their findings showed transduction efficiency of AAV2/5 to be 1.5-fold higher than AAV2/2 (based on EGFP expression) in hiPSC-RPE cells [80]. This study nicely demonstrates the relevance of determining AAV vector serotype efficiencies according to the specific cell type targeted for therapy.

Patient-derived hiPSC-RPE have been used to model and investigate other forms of inherited retinal dystrophies, such as best vitelliform macular dystrophy (BVMD) [82], gyrate atrophy (GA) [83], and a pigment retinopathy resulting from long-chain 3-hydroxyacyl CoA dehydrogenase deficiency (LCHADD) [84]. In these diseases, mutations primarily affect RPE and subsequent death and dysfunction of photoreceptors is secondary to RPE loss and/or impairment. In BVMD, disease is caused by mutations in *BEST1*, which encodes BESTROPHIN1 (BEST1) protein. BVMD hiPSC-RPE was found to be equivalent to sibling-matched hiPSC-RPE upon initial characterization until cellular stressors were introduced. When BVMD hiPSC-RPE were challenged with bovine photoreceptor outer segment (POS) over-feeding, cells accumulated autofluorescent POS waste material, expressed markers indicative of increased oxidative stress, and showed delayed RHODOPSIN degradation [82]. The function of BEST1 in RPE, currently a topic of debate, was also examined and linked to ER-mediated calcium homeostasis in this model. This conclusion is supported by the inability of BVMD hiPSC-RPE to efficiently degrade protein or transport fluid, which are calcium-dependent RPE functions [5, 82].

GA is another retinal degenerative disease that primarily affects RPE and is caused by mutations in the gene encoding the enzyme, ornithine aminotransferase (OAT). OAT works in a vitamin B_6-dependent manner to metabolize ornithine. Meyer et al. showed that OAT enzyme activity could be restored in patient-derived hiPSC-RPE with vitamin B_6 treatment [83]. GA

patients can be treated with vitamin B_6, however individual responses vary [85, 86] and the patient whose fibroblasts were isolated and reprogrammed to hiPSC for this study was nonresponsive to vitamin B_6 supplementation [73]. Meyer et al. showed that GA hiPSC-RPE OAT activity was only restored at or above a certain concentration (600 μM) of vitamin B_6 [83], demonstrating the value hiPSC technology holds for creating personalized drug potency tests using the cell type affected.

Deficiency in LCHAD is the result of a mutation in the gene encoding hydroxyacyl CoA dehydrogenase (*HADHA*), which is integral to mitochondrial fatty acid metabolism [84]. Pigment retinopathy typically manifests in LCHADD patients, however pathogenesis remains unknown. Using RPE spontaneously differentiated from LCHADD patient-derived hiPSC, Polinati et al. [84] could recapitulate the clinical disease phenotype in vitro (hypopigmented RPE) and found cells were smaller and created irregular tight junctions with neighboring cells. Furthermore, the study found cytoplasmic accumulation of neutral lipids, specifically triglycerides (TGs), indicating lipid load or inability for RPE to metabolize TGs might contribute to pathogenesis of LCHADD retinopathy [84].

The pathogenesis of AMD, despite its prevalence, is also not well understood, although oxidative stress, among other environmental and genetic factors, is thought to play a role [87, 88]. Yang et al. modeled AMD in vitro using hiPSC-RPE derived from individuals homozygous for the AMD risk alleles *ARMS2/HTRA1* (T-in/del-A) [89]. The difference between risk alleles and disease-causing mutations is important to note. It is for this reason a summary of this study has not been included in Table 2 and is instead discussed herein. To reproduce a progressing AMD phenotype in hiPSC-RPE, Yang et al. aged cells using a combination of A2E treatment and blue light exposure, creating an oxidative state in vitro [89]. Using this model, superoxide dismutase 2 (SOD2)-mediated oxidative stress response was determined to be compromised in hiPSC-RPE from AMD risk haplotype patients (T-in/del-A) as SOD2 protein activity was diminished in these cells and levels of reactive oxygen species and superoxide increased [89]. These data suggest that impaired ability to deal with increasing levels of oxidative stress in an aging retina by modulating SOD2 activity could play a role in AMD pathogenesis in individuals with risk haplotype. Treatments focused on improving SOD2 activity could have protective effects against developing AMD.

Collectively, these studies illustrate the important potential patient-derived hiPSC technology holds for, (a) accurately recapitulating RPE and retinal disease in vitro, (b) gaining insight into unknown functional aspects of disease-causing mutations and disease pathogenesis, (c) identifying unknown mutations in sporadic disease, (4) testing drugs and/or drug efficacy on affected cells, and (e) testing/optimizing targeted gene therapy using AAV vectors, among other things. HiPSC technology may also be used to better understand how RPE and other ocular tissues develop. As an example, Phillips et al. [90] created hiPSC from a patient presenting with microphthalmia caused by a mutation in the gene *VSX2/CHX10* (a neural retina-specific transcription factor). When induced to differentiate to optic vesicles, cells showed a propensity to become RPE and lagged in generating photoreceptors. Interestingly, bipolar cells were not able to be derived from the patient-hiPSCs even when VSX2/CHX10 was overexpressed [90]. Looking forward, this field of research has not only huge benefits for patients, but also the potential to broaden our knowledge of how tissues organize and develop. There is much that remains unknown about human retinal degenerative diseases and hiPSC technology combined with improved derivation methods are an exciting platform in which to study disease in vitro.

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Author Contributions

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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