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Retinal Pigment Epithelium Replacement Therapy for Age-Related Macular Degeneration: Are We There Yet?

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Abstract

Pluripotent stem cells (PSCs) are a potential replacement tissue source for degenerative diseases. Age-related macular degeneration (AMD) is a blinding disease triggered by degeneration of the retinal pigment epithelium (RPE), a monolayer tissue that functionally supports retinal photoreceptors. Recently published clinical and preclinical studies have tested PSC-derived RPE as a potential treatment for AMD. Multiple approaches have been used to manufacture RPE cells, to validate them functionally, to confirm their safety profile, and to deliver them to patients either as suspension or as a monolayer patch. Since most of these studies are at an early regulatory approval stage, the primary outcome has been to determine the safety of RPE transplants in patients. However, preliminary signs of efficacy were observed in a few patients. Here, we review the current progress in the PSC-derived RPE transplantation field and provide a comparative assessment of various approaches under development as potential therapeutics for AMD.

INTRODUCTION

Since their discovery, embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have been hailed for their ability to provide replacement tissues for a wide range of disease indications (1–4). However, the first phase I clinical trials with ESC-derived products were only recently completed (5–7). Multiple additional trials using both ESCs and iPSCs are now underway (8–10). Vision research is leading the development of this new class of replacement cell therapies, which are derived from pluripotent stem cells (PSCs). Twenty-nine retinal degeneration (RD) patients received stem cell–derived retinal pigment epithelium (RPE) transplants in six different first-in-human trials performed on three different continents (5–10). This number will likely double over the next couple of years as new data from ongoing and planned clinical trials are released (8, 9, 11, 12). The current progress in the field of PSC-derived replacement therapies for ocular diseases is a perfect example of an unmet medical need driving further advancements in a specific technology. RDs and age-related macular degeneration (AMD) are the leading causes of untreatable blindness and visual impairment, affecting millions of individuals worldwide (13, 14). The eye community capitalized on the landmark discovery that ESCs and iPSCs could be easily differentiated into one eye cell type, the RPE, the very tissue that degenerates in AMD patients and some inherited RD patients (15–19). The accessibility of the eye to minimally invasive surgical and imaging technologies helped to further advance PSC-based cell therapies for ocular diseases (20, 21). Together, advancements in stem cell biology, surgical and imaging advances in the eye, and the medical demand have positioned the eye to be one of the first organs for which a commercially approved, PSC-derived replacement therapy will likely be available soon.

Here, we present an in-depth review of PSC-derived RPE replacement cell therapy products for RDs and AMD. We discuss critical steps that determine the successful formulation of a cell therapy product and its transition from the lab to a first-in-human trial and highlight the potential roadblocks to its commercial approval. Some of the critical steps discussed here include (*a*) manufacturing the cell therapy product to match its clinical needs with quality attributes that will enable its safe and long-term integration in the eye, leading to slowing, halting, or reversing the disease course; (*b*) validating those quality attributes in vitro and in vivo in appropriate animal models; and (*c*) developing a minimally invasive delivery, and a follow-up strategy, to ensure integration success and determine the efficacy of the cell therapy product in patients.

AGE-RELATED MACULAR DEGENERATION: AN UNMET MEDICAL NEED

The choice of AMD as the first target disease for most of these stem cell trials was both serendipitous and anticipated. RPE was the first ocular cell type successfully differentiated from ESCs or iPSCs (15, 16), and it is the degeneration of RPE in AMD patients that triggers progression to advanced disease stages (19). Furthermore, previous surgical experiments transplanting a healthy RPE from the periphery of an AMD patient's eye to the same patient's macular region showed vision recovery, providing proof-of-principle that RPE cell therapy may work for such patients (21, 22). AMD is a polygenic disease induced by aging, high-calorie diet, and smoking, leading to slow degeneration of RPE cells over the lifetime of a patient (23). Therefore, no gene correction is required for any autologous or allogeneic cell therapy source. Recent work suggests that RPE cells differentiated from AMD patient iPSCs are relatively healthy when functionally validated in vitro (12) and that such healthy RPE transplants are not expected to succumb to disease processes immediately. RPE replacement therapy is also attractive from a clinical-grade manufacturing point of view. The expected clinical dose of RPE cells for AMD patients is approximately 100,000 cells, making it easier to scale up (allogeneic approach) or scale out (autologous approach) their

clinical-grade manufacturing. This small clinical dose also reduces the risk of potentially contaminating PSCs in the final cell therapy product, providing much-needed trust in PSC-derived cell therapeutic approaches.

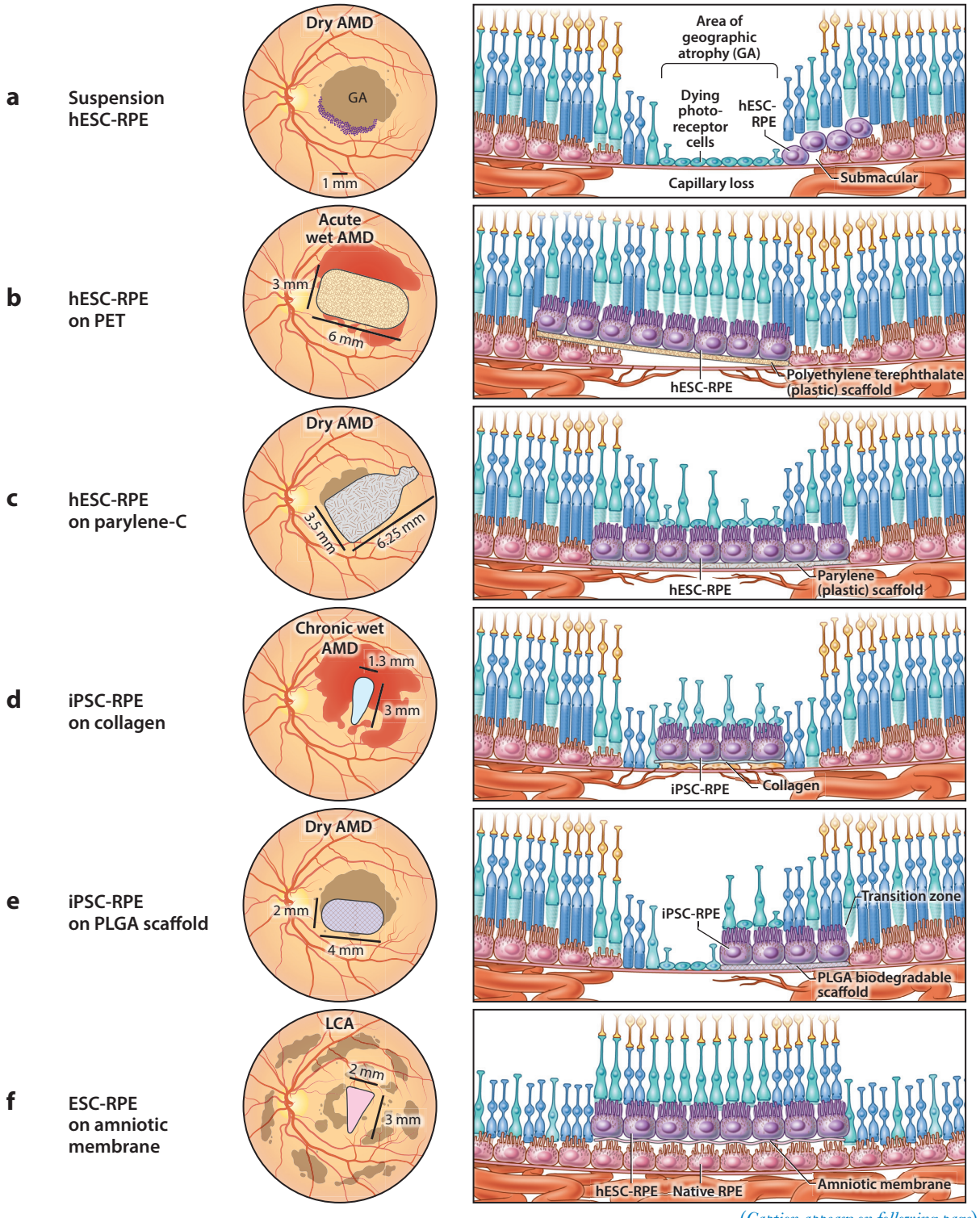
AMD, as the name suggests, is an age-onset form of RD that affects individuals mostly over the age of 55. Currently, over 200 million people worldwide are affected by AMD, with approximately 10% at an advanced disease stage (13). Since aging is one of the main risk factors for AMD, these numbers are likely to increase dramatically as life expectancy continues to rise, especially in third-world countries (24, 25). As the disease progresses, there is an irreversible loss of central vision (macular region) that results in emotional trauma and the social isolation of these patients, further increasing the cost of patient care and health management.

AMD has two advanced stages: dry AMD, or geographic atrophy, and wet AMD, or choroidal neovascularization (19). Histologically, the dry form of AMD is characterized by the death of photoreceptors in the macular region of the eye, an area with a diameter of approximately 5 mm that is responsible for our central vision (18, 24). Photoreceptors die because the RPE cells that provide them with functional support atrophy in the dry stage of AMD (19). This sequence of events suggests that transplantation of an RPE monolayer before all the photoreceptors die off may provide a potential treatment for dry AMD. This has been previously tested using a surgical procedure where a piece of RPE/choroid from the periphery of a patient's eye was cut out and transplanted into that same patient's macular region. This procedure provided visual acuity recovery in a small number of cases where the transplant engrafted well under the macula (21, 22, 26, 27). It is expected that an RPE transplant will be able to restore the native RPE monolayer, provide functional support to the photoreceptors, and prevent further photoreceptor cell death (**Figure 1**).

Like dry AMD, wet AMD also leads to severe vision loss, but the mechanism is slightly different. In an acute case of wet AMD, photoreceptors and most RPE cells are not severely damaged. However, a tear in the RPE monolayer (likely caused by a loss of some RPE cells or RPE barrier function) leads to migration of hyperproliferating choroidal vessels into the retina. These vessels leak blood or fluid, separating photoreceptors from the RPE and causing a precipitous drop in vision (28). What causes RPE tear or the loss of RPE barrier function is not clear. However, the intent of the cell therapy approach using RPE transplants is to restore the native RPE layer or patch the tear, preserving overlying photoreceptors and stopping choroidal vessels from hyperproliferating/migrating into the subretinal space. In some chronic cases of wet AMD, antivascular endothelial growth factor (anti-VEGF) antibodies have not worked well to stop the continuous blood and fluid leakage in the retina leading to fibrosis in the subretinal space. This fibrosis renders the RPE nonfunctional or even causes it to degenerate, resulting in photoreceptor cell death (28). In this case, the goal for the RPE transplant is to recreate the lost RPE, preserve leftover photoreceptors, and stop further vessel proliferation and leakage in the subretinal space by restoring physiological levels of vascular endothelial growth factor (VEGF) (**Figure 1**).

RETINAL PIGMENT EPITHELIUM: THE CELL WITH FOUR Ps (PIGMENTED, POLYGONAL, POLARIZED, AND PHAGOCYTIC)

As described above, there are several expectations for these replacement RPE transplants: Restore the missing RPE monolayer, revitalize the Bruch's membrane and the underlying choroid to reinitiate nutrient and metabolite exchange between photoreceptors and the blood supply, preserve overlying photoreceptors, repair rips in the native RPE monolayer, and stop choroidal vessels from hyperproliferating/migrating. This may sound like a tall order; however, a fully polarized native RPE monolayer can do all these functions and perhaps a lot more, utilizing the four P features (i.e., pigmented, polygonal, polarized, and phagocytic) that it has acquired developmentally (17).



(*Caption appears on following page*)

Figure 1 (*Figure appears on preceding page*)

Schematic of various ongoing and planned retinal pigment epithelium (RPE) transplant approaches, showing fundus views of the transplants (*left*) and how the transplants (*purple*) would be integrated into the subretinal space, their possible impact on retina and choroid, and the various scaffold materials involved (*right*). (*a*) Schwartz et al. (5) injected embryonic stem cell (ESC)-RPE cell suspension in the submacular region. ESC-RPE cells in suspension do not form a polarized monolayer but stay as a bolus of rounded nonpolarized cells in the submacular region. (*b*) da Cruz et al. (8) transplanted an ESC-RPE patch over the area of acute choroidal neovascularization (CNV). This 3 × 6-mm transplant was intended as an actual patch over the area of acute CNV. The RPE patch helps stop CNV and rescues photoreceptors that may not have degenerated in these specific patients. (*c*) Kashani et al. (9) used an ESC-RPE patch on a parylene scaffold, transplanted in the area of geographic atrophy (GA). Change in fixation point to over the area of the ESC-RPE patch was observed in three patients, suggesting that the RPE patch was able to recover the activity of a few photoreceptors in the transplanted region. (*d*) Mandai et al. (10) tested the first autologous iPSC-RPE patch in an acute wet AMD patient. This patch was transplanted in a macular region that was fibrotic due to chronic vessel leakage. One-year follow-up in this patient revealed the absence of new leaks. (*e*) Sharma et al. (12) propose to transplant an autologous iPSC-RPE patch using a poly-(lactic-coglycolic) acid (PLGA) scaffold at the border of the GA lesion. This patch is intended to cover parts of the transition zone where the photoreceptors are still alive to slow down or halt the expansion of the GA lesion. (*f*) Ben M'Barek et al. (11) propose to test a gelatin-embedded ESC-RPE patch on an amniotic membrane in patients with Leber congenital amaurosis (LCA). This patch will be transplanted on top of dysfunctional native RPE cells such that, over the long term, the new RPE patch will integrate into the host RPE monolayer in place of diseased cells.

RPE cells perform several functions to maintain the health and integrity of the back of the eye, including the photoreceptors, Bruch's membrane, and the choroid. RPE cells transport glucose and oxygen from the choroidal blood supply to support the high energy demand of photoreceptors and transport back to the choroid metabolites like CO_2 and lactate generated by those energetically active photoreceptors (29–31). RPE cells reisomerize the visual pigment back to 11-*cis*-retinal from its photoconverted all-*trans*-retinal isoform that is formed during light absorption by opsin proteins in the photoreceptors (32). RPE cells phagocytose photoreceptor outer segments (POSs) that are continually being damaged by light, allowing the formation of new outer segments—a process that likely depends on beta-hydroxy butyrate secreted by RPE cells (33, 34). RPE cells secrete the angiogenic and angiostatic cytokines VEGF, PEDF, IL6, IL8, and MCP-10 in a polarized fashion to maintain blood vessel confluency and permeability in the choroid (35, 36). RPE cells secrete matrix metalloproteases (MMP2, MMP9) and inhibitors of metalloproteases (TIMP1 and TIMP3) specifically on the basolateral side to help maintain Bruch's membrane conductivity for water and other metabolites (37–39). RPE cells maintain the ionic composition of the subretinal space. For instance, the potassium concentration in the subretinal space drops from 5 mM to 2 mM upon dark to light transition, leading to the activation of RPE apical membrane–localized potassium channels (e.g., Kir7.1) to correct that potassium concentration (40–42). Last but not least, RPE tissue provides a pigment shield and a physical barrier between photoreceptors and the blood supply. An ideal RPE transplant with all four Ps (pigmentation, polygonal cell shape, polarized monolayer, and phagocytic ability) should be able to perform all these tasks seamlessly for the lifetime of an individual (17).

RPE cells acquire these properties gradually during embryonic development, as they are committed to the RPE lineage from a common RPE/neuroretina progenitor cell, undergoing differentiation, cell-cycle exit, and maturation into an adult RPE cell (17, 43). In addition to expressing the genes that are required for RPE functions, for example, glucose transporter *GLUT1* (44); visual cycle genes *RPE65* and *CRALBP* (39); receptors for phagocytosis of POS *MERTK* and $\alpha\beta5$ integrins (34); pigmentation genes *TYROSINASE*, *DCT*, and *TYRP1* (43, 45); and the ion channels Kir7.1 and *CFTR* (39), these cells progressively attain their four Ps. However, the most critical among the four Ps of RPE is its polarized nature, because it is required to support most of the RPE's functions and its epithelial phenotype (46). One extreme example of changed RPE phenotype triggered by lost polarization of the monolayer is epithelial to mesenchymal transition (EMT), a process that

leads to the loss of RPE pigmentation, its polygonal shape, and its phagocytic ability (47). RPE transplants injected as a cell suspension in the subretinal space may not form a polarized monolayer and thus are at risk of undergoing EMT, affecting the long-term survival, integration, and functionality of the transplant (5, 6, 48).

Formation of a fully polarized RPE monolayer is dependent on the development of proteinaceous junctional complexes (zonula occludens and zonula adherens) between adjacent RPE cells (49). In vitro RPE cells can be induced to form tight junctions by allowing cells to form a monolayer on a variety of scaffolds (8, 9, 12). Once RPE cells attach to a substrate, they start proliferating until they reach confluency. As cells become confluent and come into contact with neighboring cells, they initiate tight-junction assembly (46). Primary cilia formed at this stage cause cells to fully exit the cell cycle and induce translocation of the canonical WNT mediator beta-catenin to the membrane, thus turning off the WNT pathway that is required for RPE proliferation (46, 50). Primary cilia induction also activates the enzyme PKC-delta that phosphorylates the myosin light chain, leading to the alignment of the actin cytoskeleton along the cell membrane and causing cells to attain their polygonal shape while further strengthening tight junctions (46). After four to five weeks in culture, these junctional complexes mature to the point where apical and basolateral membranes of RPE cells are mostly isolated and the paracellular space between neighboring RPE cells is sealed (51). Completion of this process can be measured as the transepithelial/barrier resistance of the RPE monolayer, which for a healthy, fully mature RPE monolayer should be several hundred Ohms per square centimeter (12, 46, 52). Barrier resistance is a key feature used by some groups to provide evidence of the maturation and polarization of PSC-derived RPE transplants (10, 12).

STEM CELL–DERIVED RETINAL PIGMENT EPITHELIUM

As mentioned above, RPE was one of the first ocular tissues differentiated from ESCs, which was noted in seminal publications by Haruta et al. (15), Kawasaki et al. (16), and Klimanskaya et al. (53). Since this initial discovery, several groups have reported an easy method to differentiate RPE from both ESCs and iPSCs using the spontaneous differentiation process, where stem cells are differentiated by merely changing their culture medium to one without any specific growth factors (**Table 1**). After 20–25 weeks, multiple tiny pigmented colonies emerged out of these differentiating cultures (8, 9, 54, 55). Since the process relies on the spontaneous ability of PSCs to differentiate into cells of all three germ layers, the efficiency of this method is rather low (<10%) and requires expansion of these pigmented colonies to obtain sufficient pure cells for preclinical and clinical needs (8, 9, 54, 55). Nevertheless, published work has validated at least some RPE functions (**Table 1**) in cells differentiated from ESCs and iPSCs using this protocol (8, 9, 11). All four studies involving ESCs have utilized this manufacturing process with slight differences in the manufacturing timeline (**Table 1**). It is worth mentioning that the effect of this different timeline on the quality of the RPE transplant is not apparent, as no direct comparisons between different products have been performed.

Directed or developmentally guided differentiation of RPE from PSCs utilizes the knowledge of the embryonic development of RPE cells (12, 56–62). Most of the early observations of developmental pathways for RPE differentiation come from *Xenopus*, chick, and mouse work. For instance, the role of IGF in the induction of anterior neuroectoderm precursors to the eye-field cells was first established in *Xenopus* (63). The role of TGF-beta (Activin A) in RPE specification was first shown in a chick study and was later reproduced in human ESCs (59, 64). The importance of WNT in RPE differentiation was initially demonstrated in mouse models and later reproduced in human ESCs/iPSCs (12, 50, 65, 66). It is important to note that the requirement of these growth

Table 1 Summary of manufacturing process for ongoing and planned PSC-derived RPE transplant studies

Reference(s)	PSC source	PSC-RPE differentiation method (timeline)	Differentiation procedure	Transplant functional validation	Validation procedure
Schwartz et al. (5, 6) Song et al. (7)	ESC (MA09), allogeneic	Spontaneous (timeline NA)	1. ESC grown on mouse feeder 2. RPEs at second passage were used for transplantation	RPE validated before purification and banking	1. IF and qRT-PCR for PAX6, ZO1, BEST1, and MITF 2. Phagocytosis, flow cytometry using phRODO particles 3. Melanin content measured spectrophotometrically 4. Lacking OCT-4 ICC and alkaline phosphatase staining
Mandai et al. (10) Osakada et al. (62, 68)	Autologous iPSCs	Directed/ spontaneous differentiation (10 months)	1. iPSC grown on patient-derived skin fibroblasts 2. iPSCs induced to neuroectoderm using Y-27632, SB431542, and CKI-7 3. Neuroectoderm spontaneous differentiation into RPE 4. Pigmented colonies manually isolated and kept as suspension culture for 10 days 5. Microscopically isolated colonies seeded and expanded with SB431542 and FGF2 6. Third-passage cells seeded on collagen-coated transwell and maintained in FGF2 + SB431542 7. Collagenase IV treatment to lift iPSC-RPE patch	iPSC-RPE patch validation	1. Purity by BEST1/PAX6 2. >95% by immunostaining 3. TER (290 Ohm/cm^2) 4. PEDF, VEGF secretion 5. IF and qRT-PCR for RPE markers (BEST1, RPE65, MERTK, CRALBP) 6. TEM 7. Phago, FITC porcine POS
Kashani et al. (9) Pennington et al. (90)	ESC (H9), allogeneic	Spontaneous differentiation (32 weeks)	1. Spontaneous differentiation for 16 weeks on Synthemax-coated plates 2. Pigmented patches manually picked and dissociated using TrypLE and seeded on VTN-coated plates 3. Passaged 3 times in 3 months 4. Passage 3 cells seeded on VTN-coated parylene scaffold and matured for 28–35 days	Passage 2 cells validated	1. IF (ZO1, PMEL17, OTX2) and qRT-PCR (RPE65, TYR, BEST1, TYRP1, PMEL17) for RPE markers 2. Lacking OCT4 and SALL4 expression 3. POS phagocytosis 4. Polarized PEDF secretion
da Cruz et al. (8)	ESC (SHEF01), allogeneic	Spontaneous differentiation (30–58 weeks)	1. Up to 22 weeks of spontaneous differentiation on VTN-coated plates 2. Pigmented patches manually picked and expanded on CELLstart-coated plates for 5–16 weeks 3. RPE cells matured on PET membrane for 3–20 weeks	PET-RPE patch validation	1. Visual inspection 2. Loss of Lin28 3. PMEL17 ICC 4. Lacking Lin28 expression 5. PEDF secretion 6. Phagocytosis of labeled POS by IF
Ben M'Barek et al. (11)	ESC (RC-9), allogeneic	Spontaneous differentiation (17 weeks) ES line derived on fibroblast, later made feeder free using a commercially available substrate	1. Up to 5 weeks of spontaneous differentiation on CELLstart-coated plates 2. Pigmented patches manually picked and expanded on CELLstart-coated plates for another 5 weeks 3. Isolated pigmented patches expanded for 3 weeks 4. Cells matured on human amniotic membrane for 6 weeks	hAM-RPE patch validation	1. IF for MITF, TYRP1, ZO1, EZRIN, BEST1 2. TEM, SEM 3. Secretion of VEGF 4. RT for PAX6, BEST1, RPE65, MITF, OCT4, and Nanog 5. Porcine-labeled POS phagocytosis by IF

(*Continued*)

Table 1 (*Continued*)

Reference(s)	PSC source	PSC-RPE differentiation method (timeline)	Differentiation procedure	Transplant functional validation	Validation procedure
Sharma et al. (12)	Autologous iPSCs	Directed differentiation (monolayer culture, 11 weeks)	1. iPSCs grown on VTN 2. iPSCs induced to optic neuroectoderm using LDN-193189, SB431452, CK1–7, PD0325901, IGF-1 for 12 days 3. Optic neuroectoderm induced to committed RPE using activin and nicotinamide for 10 days 4. Committed RPE growth phase of 22 days 5. Enrichment of RPE progenitors using CD24 and CD56 antibodies and maturation on PLGA scaffolds for 5 weeks	PLGA-iRPE patch validation	1. Purity by flow for PMEL17, TYRP1, BEST1, CRALBP 2. Lacking OCT4, SSEA4, TRA1–81 by flow cytometry 3. SEM, TEM 4. IF for RPE65, GPNMB, COLIV, COLVIII 5. qRT-PCR for RPE65, BEST1, GPNMB, MYRIP, TYRP1, ALDH1A3, TRPM1, OCA2 6. Shape metrics 7. TER (>400 Ohm/cm^2) 8. VEGF secretion 9. POS phagocytosis

Abbreviations: ES, embryonic stem; ESC, embryonic stem cell; FGF2, fibroblast growth factor 2; ICC, immunocytochemistry; IF, immunofluorescence; IGF-1, insulin-like growth factor-1; iPSC, induced pluripotent stem cell; NA, not applicable; PEDF, pigment epithelium derived factor; PET, polyethylene terephthalate; PLGA, poly-(lactic-coglycolic) acid; POS, photoreceptor outer segment; PSC, pluripotent stem cell; qRT-PCR, quantitative real time-polymerase chain reaction; RPE, retinal pigment epithelium; SEM, scanning electron microscopy; TEM, transmission electron microscopy; TER, transepithelial resistance; VEGF, vascular endothelial growth factor; VTN, vitronectin.

factors makes it challenging to optimize good manufacturing practice (GMP)-compatible protocol for a directed differentiation process, as compared to spontaneous differentiation that does not require an extensive set of reagents. However, directed differentiation has several advantages over spontaneous differentiation: It is faster (10 weeks compared to 25 weeks for spontaneous differentiation), thus reducing the cost of GMP-compatible manufacturing (8, 12). It is also more efficient (60–80% as compared to <10% for spontaneous differentiation), making it easier to generate pure cells without the need for additional cell expansion (12, 62, 66–68). Minimal expansion of RPE cells helps preserve their epithelial phenotype (69), increases their reproducibility in autologous cell therapy approaches, and preserves epigenetic modifications on gene promoters and enhancers, thus generating RPE cells that more closely resemble native RPE cells in their functional readouts (12, 70). Although these in vitro observations about RPE transplants manufactured using directed differentiation seem promising, at present, there is limited evidence that these cells perform better in vivo in animal models or patients (12).

FUNCTIONAL VALIDATION OF RETINAL PIGMENT EPITHELIUM TRANSPLANTS

In addition to purity and sterility, the functionality of a cell therapy product is another critical quality attribute that will likely determine its success. As discussed above, several structural and functional properties of the RPE monolayer have been well studied and can be measured in vitro to validate an RPE transplant. Several published preclinical and clinical studies have used structural and functional validation assays, including simple visual inspection of the RPE monolayer, its confluency, and its pigmentation (8); expression of RPE markers (10, 12); barrier or transepithelial resistance (12, 71); functional intracellular calcium signaling (71); polarized cytokine secretion (9, 12); ability to phagocytose POS (9, 12); ability to maintain ionic composition in the subretinal space (12, 71); and the ability to transport water from the apical to basal sides (**Table 1**). It is, however, essential to note that most of these RPE functions are dependent on an intact monolayer, making it harder to functionally validate RPE cell suspension as a clinical product.

Measurements of select gene expression patterns or visual inspection of the RPE monolayer alone, although simple, may not be sufficient to provide complete insight into RPE physiology or be able to address batch-to-batch or donor-to-donor variability in manufacturing runs. Capturing such variability can provide insight into the potential safety of the transplant and is required to validate manufacturing runs. Miyagishima et al. (71) demonstrated that RPE derived from different iPSC lines may contain several RPE-like features, including robust pigmentation, apparent cobblestone morphology, and RPE-specific gene expression, but showed differences in the output of purinergic receptor signaling when measured using intracellular calcium store activity and fluid flow from the apical to basal side of the RPE monolayer. Purinergic signaling is critical for in vivo RPE functions; light-stimulated ATP released by the photoreceptors causes the activation of purinergic receptors on the RPE apical surface. This leads to calcium release from intracellular stores, driving the activation of basolateral chloride channels and closing the apical K channels and fluid flow from the subretinal (apical) space into the choroid (basal) (71, 72). The inability of RPE to transport fluid leads to a condition called macular edema in which fluid accumulates between the photoreceptors and RPE and separates the two cell types, resulting in photoreceptor death and vision loss (72, 73).

In another example, Sharma et al. (12) recently determined batch-to-batch and donor-to-donor variability of patient-specific iPSC-RPE cells using a systematic analysis of RPE shape metrics, gene expression, polarized cytokine secretion, barrier resistance, and the ability to phagocytose POS. These data revealed that the donor-to-donor variability is more prominent compared to the clone-to-clone variability in different iPSC-RPE manufacturing runs. Going forward, such approaches are critical for validating RPE or any cell therapy products. Furthermore, such functional validation will also help identify a potency assay that is required before commercial approval of any cell therapy product (**https://www.ecfr.gov/**).

TOXICOLOGY OF RETINAL PIGMENT EPITHELIUM TRANSPLANTS

There are five major safety concerns for any cell therapy product, which are discussed in detail below: the presence of infectious agents such as bacteria, fungi, mycoplasma, and viruses; the presence of foreign substances (endotoxin and animal products); the presence of contaminating PSCs; the presence of cells with undesired cell lineages; and the generation of cell therapy products that are genomically unstable.

First, the sterility of a cell therapy product and of all its animal origin reagents is monitored at key banking/cryopreservation stages and in the final formulation, as per Title 21 Section 610.12 of the Code of Federal Regulations (CFR) guidance document from the Food and Drug Administration (FDA) (74). All clinical programs described here have validated their processes for the manufacturing of a sterile product. There is one fundamental difference between allogeneic and autologous cell therapy products for the testing of adventitious viruses. All allogeneic products must be tested for the entire panel of adventitious viruses (5) to rule out the possibility that a virus from a contaminated product spreads to patients that receive a contaminated allogeneic transplant. Because autologous products are delivered back to the patients they are derived from, they do not require this extensive viral testing.

Second, the presence of bacterial (endotoxins) or animal proteins could induce an immune response against the cell therapy product, leading to transplant rejection and severe adverse events. Bacterial endotoxins, like lipopolysaccharides and lipoglycans, come from the outer membrane of gram-negative bacteria and can contaminate the cell therapy product through plasticware, reagents, serum, or even water (75). As per CFR Title 21 Section 610.13(b) (74), the acceptable endotoxin limit is 0.2 EU/kg body weight/dose. It is expected that all these clinical programs have validated their manufacturing processes to meet the FDA acceptance limit for endotoxins.

Third, contamination of PSC-derived therapeutics by PSCs has been a significant safety concern, because when PSCs can proliferate and differentiate unchecked, they can lead to teratoma formation. A minimum of 500 ESCs is required to form a teratoma when subcutaneously injected as pure cells in mice (76). This amounts to 0.5% of the clinical doses of 100,000 cells that are currently being used in most RPE transplants. In comparison, Schwartz et al. (5, 6) demonstrated that even 1% ESCs mixed with RPE cells in immunocompromised mice did not lead to any teratoma formation in the subretinal space (**Table 2**). The concern of PSC contamination in RPE transplants is further undermined by a recent demonstration that PSCs cannot survive the culture conditions used for RPE differentiation (12). In an in vitro spiking study, 0.01%, 0.1%, 1%, and 10% iPSCs mixed with RPE cells were seeded on scaffolds under RPE maturation conditions. In less than 14 days, all iPSCs died, as determined by flow cytometry and gene expression analysis, confirming the notion that PSCs cannot survive RPE differentiation conditions. Similar evidence supporting the absence of PSCs in the final RPE transplant has been obtained by most groups (8–10) (**Table 2**). Combined, the current data provided by these clinical studies seem sufficient to alleviate this major safety concern for PSC-based therapies.

Fourth, non-RPE cells and non-PSCs that may contaminate the RPE transplant and RPE cells that change phenotype after transplantation can both be detrimental for an RPE transplant. For instance, in the case of RPE cell suspension transplants, exposure to inflammatory cytokines in a diseased environment can induce EMT in transplanted cells, causing cells to lose their RPE phenotype, become fibroblastic, and form membranous scars that can lead to severe vision loss. Such micromembranes were detected in some patients that received RPE cells in suspension in the Schwartz et al. study (5, 6). For RPE transplants delivered as a patch, EMT is not a major concern because the patch structure allows cells to exit the cell cycle and become fully polarized, thus reducing the possibility of EMT.

Fifth, two landmark publications have recently highlighted the issue of PSC genomic instability. Merkle et al. (77) showed enrichment of p53 mutations in ESCs or iPSCs cultured for more extended periods. Although most cell lines tested in this study were generated under non-GMP-compliant conditions, this observation is supported by previous work showing that cells in culture tend to accumulate genomic alterations that provide a survival advantage (78, 79). Mandai et al. (10) reported copy number variations in clinical-grade iPSCs reprogrammed from AMD patients' skin fibroblasts. Genomic alterations can be acquired in iPSCs during the reprogramming process or can be enriched from preexisting genetic mosaicisms in source cells (80). Irrespective of the origin, if potentially cancerous changes are enriched in a cell therapy product, they are not desirable. Recent work suggests that oncogenic mutations may be avoided by using CD34+ cells as a starting source for iPSCs (12) (**Table 1**). This may be since CD34+ cells are progenitor cells in nature and continue to retain their proliferative potential when expanded in vitro (81). Furthermore, as demonstrated by Sharma et al. (12), oncogene exome sequencing, along with standard G-band karyotyping, may provide relevant information to support the safety of cell therapies derived from iPSCs.

Most preclinical studies performed to date have tested the tumorigenic, toxicity, and migratory profiles of PSC-derived RPE cells, in addition to confirming their sterility and lack of endotoxins (5, 6, 8–12). Immunocompromised mice or rats are used to ensure that transplanted cells survive long enough to reveal their tumorigenic potential (**Table 2**). A detailed discussion of GLP preclinical study design is out of the scope of this review, but it is important to note a fundamental difference in study designs between allogeneic and autologous cell therapies. For allogeneic products, the safety of the clinical product is tested, whereas for autologous products, the safety of the clinical manufacturing process is tested. For example, with an allogeneic product, a clinical batch of the cell therapy product is tested, but for autologous cell therapy products, only cells made from

Table 2 Summary of preclinical studies performed to test the safety and feasibility of RPE transplants

Reference(s)	Animal model	Study purpose	Dose	Study conclusion(s)
Schwartz et al. (5)	NIH-III immune-compromised mice	Tumorigenicity	50,000 and 100,000 hESC-RPE	No human-origin tumor detected No Ki67 positivity detected in hESC-RPE
		Tissue distribution	Spiking of 0.01%, 0.1%, and 1% ESCs in 100,000 ESC-RPE	No cells found outside the eye
da Cruz et al. (8)	NIH-III immune-compromised mice	Tumorigenicity	6×10^4 hESC-RPE	No human-origin tumor detected No proliferation No migration
	Healthy pigs	Feasibility, tissue distribution, and safety	3 × 6-mm RPE patch	Photoreceptor survival over RPE patch
Kashani et al. (9) Koss et al. (91)	Healthy pigs	Feasibility and safety	3.5 × 6-mm RPE patch	None of the animals had high IOP 75% of pigs with good implant placement No inflammation or migration of RPE cells
Mandai et al. (10) Kamao et al. (70) Kanemura et al. (92)	Healthy cynomolgus monkey	Survivability and safety	1 × 2-mm iPSC-RPE patch	No human-origin tumor detected Graft rejection in allotransplants Autotransplants survived for 12 months Dislodged RPE cells noted in vitreous
	NOG-SCID mice	Tumorigenicity and subcutaneous transplants	1×10^6 cells	
	Nude rats	Tumorigenicity and subretinal transplants	1 × 1-mm patch	
Sharma et al. (12)	Nude rats	Tumorigenicity and toxicity	iPSC-RPE patch (0.5 mm^2), iPSC-RPE suspension (100,000 cells)	No human-origin tumor or teratoma No systemic toxicity
	Pigs with laser-injured RPE	Feasibility and efficacy	iPSC-RPE sheet (2 × 4 mm), iPSC-RPE suspension (100,000 cells)	iPSC-RPE patch integration No degeneration of photoreceptors over RPE patch Human RPE phagocytosing pig POS
Ben M'Barek et al. (11)	Athymic nude rats	Feasibility	ESC-RPE patch (2–3 mm^2) ESC-RPE suspension (50,000 cells)	Both suspension and patch cells expressed human markers

Abbreviations: ESC-RPE, embryonic stem cell–retinal pigment epithelium; IOP, intraocular pressure; iPSC-RPE, induced pluripotent stem cell–retinal pigment epithelium; POS, photoreceptor outer segment.

one or more patients and manufactured using the clinical process are used. These cells may or may not be from the patients who will be enrolled in the transplantation trial, but the manufacturing of the clinical product is performed using the clinical-grade manufacturing process planned for the clinical trial. All studies performed so far have demonstrated the safety profile of their respective RPE transplants (5, 6, 8–12).

EFFICACY OF RETINAL PIGMENT EPITHELIUM TRANSPLANTS

The goal of these cell therapy products is to replace degenerated RPE with a young and healthy PSC-derived RPE. An ideal animal model would replicate the disease pathology of AMD and allow for testing RPE transplants as replacement tissue. Currently, no such model exists. Most efficacy studies have used the Royal College of Surgeons (RCS) rat model, where dysfunctional RPE cells stimulate RD. RCS RPE cells cannot phagocytose POS due to a mutation in a gene, *MERTK*, involved in outer segment phagocytosis (8, 82, 83). One of the caveats with the RCS rat model is that the RPE does not degenerate, and the success of a transplant is assessed by its ability to clear out POS debris that accumulates in the mutant model. Another major problem is that even a sham surgical intervention can provide photoreceptor protection (84). Despite such caveats, careful analysis shows that sham/saline/other nonspecific effects in RCS rats do not last over the long term (85, 86). Thus, RPE transplant activity can be tested at longer-term timepoints in the RCS rat model, and most of the stem cell–based therapies reviewed here have utilized this model to demonstrate the efficacy of their cell therapy products (5, 6, 8–12).

Feasibility and safe delivery of a human clinical dose of the RPE patch have been tested in pigs and monkeys using a surgical procedure and delivery tools similar to those planned for human surgeries (8–10, 12, 87) (**Tables 2** and **3**). However, until recently, the efficacy of a human clinical dose of an RPE transplant was not tested in any large animal model. Sharma et al. (12) published a laser-induced RPE injury in a pig model that displayed photoreceptor degeneration triggered by laser-induced RPE cell death and thus recapitulated some features of AMD pathology. A 2 × 4-mm human iPSC-RPE patch was transplanted into a laser-injured pig eye before the photoreceptors degenerated, and the RPE patch integrated into the back of the eye within ten weeks, began phagocytosing the POS, and limited the death of overlying photoreceptors (**Table 2**). In a comparative analysis performed using an equal number of iPSC-RPE cells in suspension, Sharma et al. found the suspension to be less potent in rescuing laser-damaged pig photoreceptors, supporting the hypothesis that a monolayer RPE patch is more effective than iPSC-RPE suspension in rescuing photoreceptor degeneration.

CLINICAL DOSE AND DELIVERY PROCEDURE

Scaffolds used by various groups for RPE patch delivery are likely neutral in their pharmacological properties, but they do indirectly provide a therapeutic benefit (**Figure 1**, **Tables 1** and **3**). The scaffold aids in RPE monolayer polarization and its delivery as a patch. Furthermore, a scaffold's positive or negative effect on disease etiology cannot be completely ruled out. For instance, it is possible that the scaffold provides immune protection to the allo-ESC-RPE products, but fibrotic alterations around the plastic scaffold that accumulate over time and alter long-term functioning of the transplant cannot be ruled out (8, 9). A biologically compatible scaffold that replaces Bruch's membrane may solve the problem of long-term fibrosis. Three such choices have been tested: collagen coating without any scaffold (10), an amniotic membrane that is used in corneal surgeries (11), and a biodegradable scaffold made of poly-(lactic-coglycolic) acid (PLGA) polymer (88). This PLGA scaffold has been shown to help RPE form a confluent, polarized monolayer such that the cells secrete their own extracellular matrix and form a Bruch's membrane–equivalent structure

Table 3 Comparative assessment of transplantation devices used for delivery of RPE transplants

Reference(s)	Device name	Type of transplant	Cannula size	Implant size (dosage)	Tool operation
Schwartz et al. (5, 6)	MedOne PolyTip® cannula	Cell suspension	25 G	150 uL Cohort 1: 50,000 cells Cohort 2: 100,000 cells Cohort 3: 150,000 cells	VFI
da Cruz et al. (8)	Introducer tool	Rolled RPE patch	NA	3 × 6 mm PET scaffold (~100,000 cells/patch)	Manual, rotary control
Kashani et al. (9) Fernandes et al. (87)	Tissue injector	Rolled RPE patch	17 G	3.5 × 6.25 mm Parylene scaffold (~125,000 cells)	Manual, push button
Mandai et al. (10)	Custom surgical device	Flat RPE patch (no scaffold)	20 G modified intravenous cannula	1.3 × 3 mm No scaffold (<4,500 viable cells/mm^2)	Manual, plunger
Sharma et al. (12)	Transplantation tool	Flat RPE patch	2.5 mm	2 × 4 mm PLGA scaffold (~75,000 cells/patch)	VFI
Ben M'Barek et al. (11)	Tissue injector	Rolled RPE patch (gelatin embedded)	NA	2 × 3 mm hAM scaffold (~50,000 cells/patch)	Manual, plunger

Abbreviations: hAM, human amniotic membrane; G, gauge; NA, not available; PET, polyethylene terephthalate; PLGA, poly-(lactic-coglycolic) acid; RPE, retinal pigment epithelium; VFI, viscous fluid injector.

(12, 88). Over time, the PLGA completely degrades, allowing the RPE patch to integrate with the pig eye. It is not known which, if any, of these scaffold approaches will work in the longer term, but the use of a scaffold does allow transplanting the RPE monolayer as tissue that may easily integrate into the eye and hit the ground running.

In addition to the starting cell source and the method of RPE differentiation, the surgical tool and the clinical dose used in these studies are other critical variables (5, 6, 8–12). **Table 3** summarizes various approaches used for the delivery of RPE cells in suspension or as a patch. The injection of cell suspension is certainly a more straightforward and less invasive procedure, requiring an injection cannula as compared to transplantation of a patch, which in many cases requires folding a large RPE patch 6 × 3 mm in size and unrolling it in the subretinal space (8), embedding the amniotic membrane patch in gelatin to provide strength during surgery (11), or a large sclerotomy (2.5 mm) (12) (**Table 3**). The tool design significantly differs between procedures depending upon the delivery approach; some require holding the end of the patch to roll it before transplantation (8) (**Table 3**). In any case, the patch transplant procedure is surgically more invasive, may require longer recovery times, and often also results in a scar on the retina. At this stage, it is not clear if the advantages of transplanting an RPE patch outweigh the disadvantages of a more invasive surgical procedure. Longer-term studies with more patients in each category will provide the much-needed data to make this assessment.

PATIENT STUDIES

RPE derived from ESCs has been tested in 29 patients, and only one patient so far has been transplanted with RPE derived from iPSCs (5, 6, 8–10, 12). Seven patients have received an RPE patch

(six on plastic scaffolds and one without any scaffold), and 22 patients received RPE suspension [11 with AMD and 11 with Stargardt's disease (STGD)]. Although this patient population is too small to make a comparative analysis of the data collected from these five clinical studies, the safety of RPE transplantation is confirmed by all of these studies. No severe adverse effects were seen in any of the patients, and most of the adverse effects were related to either the immunosuppression regimen or the dosing procedure (**Table 4**).

Table 4 Summary of patient outcomes for ongoing and planned pluripotent stem cell–derived RPE transplant studies

Reference(s)	Patient population	Follow-up	Outcomes	Adverse events
Schwartz et al. (5, 6) Song et al. (7)	Dry AMD: 11 patients (70–88 years), BCVA 20/200 hand motion STGD: 11 patients (20–71 years)	Median 22 months	AMD: median VA increase treated versus untreated eye (14 letters versus 1 letter, $P = 0.0117$) STGD: trend toward improved VA in treated eye (12 letters); no adverse proliferation	One eye developed endophthalmitis One eye developed vitreous inflammation that resolved in 6 months Three eyes developed preretinal patches, noncontractile Four eyes developed cataract
Mandai et al. (10)	One wet AMD patient (77 years) BCVA 20/200 right eye	1 year	No evidence of leakage or recurrence of hemorrhage No need for additional anti-VEGF injections BCVA maintained at 20/200 Fixation shifted closer to the transplant	Choroidal hemorrhage 3 days post transplantation Scaffold curling Cystoid macular edema
Kashani et al. (9)	Dry AMD: 5 patients (69–85 years) Visual acuity on ETDRS chart (range 3–22)	4–12 months	Three patients VA stable One patient showed 17-letter improvement All four patients had ELM detectable by OCT over the area of the transplant Three patients had fixation detected over the transplant	One patient could not be transplanted Mild to moderate surgery-associated hemorrhage was noted in all four patients; it resolved in three without intervention, and one required anti-VEGF injections
da Cruz et al. (8)	Acute wet AMD: 2 patients (60 and 84 years) Visual acuity on ETDRS chart (10 and 8, respectively)	4–12 months	Patient 1 and Patient 2 had 19- and 21-letter improvements, respectively RPE cell migration off the patch Fixation changed to the area of the patch	Exposure of the suture for fluocinolone implant in Patient 1 Worsening of diabetes in Patient 2 PVR with tractional membranes in Patient 2

Abbreviations: AMD, age-related macular degeneration; BCVA, best corrected visual acuity; ELM, external limiting membrane; ETDRS, early treatment diabetic retinopathy study; OCT, optical coherence tomography; PVR, proliferative vitreoretinopathy; RPE, retinal pigment epithelium; STGD, Stargardt's disease; VA, visual acuity; VEGF, vascular endothelial growth factor.

The target population consisted of mostly advanced AMD patients, including 15 dry AMD patients, 11 STGD patients, and 3 wet AMD patients (5, 6, 8–10) (**Table 4**). Because of the design of a phase I safety study, most patients had extremely poor visual acuity. Several patients could only see hand motions—not much improvement in vision is anticipated from patients with such poor vision. Importantly, none of the patients experienced a further decline in vision. It is important to note that none of the three wet AMD patients required additional anti-VEGF injections, suggesting that RPE transplants were able to stop choroidal vessels from growing into the retina. For the two acute wet AMD patients that received the ESC-RPE patch, a significant improvement in visual acuity was noted (8). It is not clear at this stage whether this improvement can be attributed solely to the patch or if the surgery itself also provided some benefit. Previous work has suggested that a subretinal surgery that cleans out blood could improve visual acuity in such patients (89). In the case of dry AMD, any recovery in visual acuity seen after transplantation is likely due to RPE transplants, as was seen in at least one of the patients that received an RPE patch (**Table 4**). At this early clinical stage, it is difficult to determine the actual mechanism for RPE transplant potency. As some of these trials advance to commercial stages, the mechanistic insights into the potency of RPE transplantation will become imperative.

ROADBLOCKS FOR COMMERCIAL APPROVAL

Ongoing phase I safety trials have provided enough evidence to confirm the safety of these potential therapeutic approaches. Although there are some signs of efficacy, most of these studies are in the early stages, while the targeted patient population is at an advanced disease stage, making it challenging to ascertain more substantial claims of efficacy. The potency of these transplants will be more convincingly tested in phase II and III clinical trial stages with a larger patient population, perhaps with better starting visual acuity where both vision improvement and worsening are possible. Long-term integration and survival of PSC-derived RPE as a functional monolayer will likely govern transplant success. As discussed above, the manufacturing process and subretinal delivery of the transplant may factor in its long-term integration and survival. Phase II and III clinical trials will require the availability of RPE transplants across multiple clinics. This can only be achieved easily by using a cryopreserved transplant. For RPE cells in suspension, steps to cryopreserve and thaw the clinical product have been optimized (5). Cryopreservation and the revival of the RPE patch as a final functional product will need to be worked out to overcome these challenges to the commercialization of RPE transplants. With the 21st Century Cures Act signed by President Obama, an accelerated path for the approval of such advanced cell therapy products is now in sight.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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