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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 source for replacement tissues 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. Several recently published clinical and preclinical studies have tested PSC-derived RPE as a potential treatment for AMD. To date, multiple approaches have been taken to manufacture RPE cells from PSCs, to functionally validate differentiated RPE cells *in vitro* and *in vivo*, 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 of the clinical studies has been to establish the safety profile of RPE transplants in patients. However, preliminary signs of efficacy were observed in some patients. Here, we review the current progress in the PSC-derived RPE transplantation field and provide a comparative assessment of various therapeutic approaches under development as potential cell therapies for AMD.

Keywords

Pluripotent stem cells; embryonic stem cells; induced pluripotent stem cells; transplantation; good manufacturing practice; Cell Therapy; retinal pigment epithelium; AMD; retinal degeneration

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 all disease indications possible (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 – derived from pluripotent stem cells (PSCs). Twenty-nine retinal degeneration patients received stem cell derived RPE transplant in six different

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first-in-human trials performed in over 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 will be released (8, 9, 11, 12). The current progress in the field of PSC-derived replacement therapies for ocular diseases is a perfect example where an unmet medical need drove further advancements in a technology. Retinal degenerations (RDs) and age-related macular degeneration (AMD) are the leading causes of untreatable blindness and visual impairment, affecting millions of individuals world-wide (13, 14). The eye community capitalized on the landmark discovery that ESCs and iPSCs could be "easily" differentiated into one eye cell type – the retinal pigment epithelium – 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 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 where 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 determined 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: **(1)** 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; **(2)** validating those quality attributes *in vitro* and *in vivo* in appropriate animal models; and **(3)** developing a minimally invasive delivery, and a follow up strategy, to determine integration and 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 has been 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 the 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 patients' 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 with a substantial contribution of aging, diet, and smoking, leading to slow degeneration of RPE cells over the life time 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 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 a much needed “trust” in PSC-derived cell therapeutic approaches.

AMD, as the name suggests, is an age-onset form of retinal degeneration 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 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 – a ~5 mm diameter area that is responsible for our central vision (18, 24). Photoreceptors die because 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 patients’ 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, causing a precipitous drop in vision (28). What causes RPE tear or loss of RPE barrier function isn’t clear. However, the cell therapy approach using RPE transplants is to restore the native RPE layer or “patch” the tear, preserve overlying photoreceptors, and stop choroidal vessels from hyperproliferating/migrating into the sub-retinal space. In some chronic cases of wet AMD, where anti-vascular endothelial growth factor (anti-VEGF) antibodies have not worked well to stop the continuous blood completely and fluid leakage in the retina that leads to fibrosis in the sub-retinal space. This fibrosis renders the RPE non-functional 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 left-over photoreceptors, and stop further vessel proliferation and leakage in the sub-retinal space by restoring physiological levels of VEGF (Figure 1).

RETINAL PIGMENT EPITHELIUM – THE CELL WITH FOUR ‘P’S (PIGMENTED, POLYGONAL, POLARIZED, AND PHAGOCYTIC):

As described above, there are several expectations from these replacement RPE transplants: restore the missing RPE monolayer; revitalize the Bruch’s membrane and the underlying choroid - to re-initiate 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 that it has acquired developmentally (17).

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 re-isomerize 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 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 angiogenic and angiostatic cytokines VEGF, PEDF, IL6, IL8, MCP-10 in a polarized fashion to maintain the 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 sub-retinal space. For instance, the potassium concentration in the sub-retinal 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., Kir 7.1) 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 ‘P’s - 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, CRALBP (39), receptors for phagocytosis of POS - MERTK alphavbeta5 integrins (34), pigmentation genes TYROSINASE, DCT, TYRP1 (43, 45), and the ion channels Kir7.1 and CFTR (39), these cells progressively attain their four ‘P’s. However, the most critical ‘P’ among the four P’s of RPE is their polarized nature, because it is required to support most of RPE 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 loss of RPE pigmentation, a polygonal shape, and phagocytic ability (47). RPE transplants injected as a cell suspension in the sub-retinal space may not form a polarized monolayer and thus are at risk of undergoing EMT affecting 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 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 an enzyme PKC-delta that phosphorylates the myosin light chain leading to the alignment of the actin cytoskeleton along the cell membrane causing cells to attain their polygonal shape while further strengthening tight junctions (46). After 4–5 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 trans-epithelial/barrier resistance of RPE monolayer, which for a healthy fully-mature RPE monolayer should be several hundred Ohms.cm^2 (12, 46, 52). Barrier resistance is a key feature used by some groups to provide evidence of maturation and polarization of PSC-derived RPE transplants (10, 12).

STEM CELL DERIVED RPE:

As mentioned above, RPE was one of the first ocular tissue differentiated from ESCs; noted in a seminal 2004 publication (15, 16, 53). Since this initial discovery, several groups have reported an instead “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 a medium without any specific growth factors (Table 1). After 20–25 weeks, multiple tiny pigmented colonies emerge 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 embryonic development of RPE cells (12, 56–62). Most of the early observations of developmental pathways for RPE differentiation comes 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 factors makes it challenging to optimize GMP-grade manufacturing for a directed differentiation process, as compared to spontaneous differentiation that doesn't require an extensive set of reagents. However, directed differentiation has several advantages over spontaneous differentiation: it is faster (10 weeks as compared to 25 weeks for spontaneous differentiation), thus reducing the cost of GMP-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 maintain their epithelial phenotype (69), increases their reproducibility in autologous cell therapy approaches and preserves epigenetic modifications of 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 no evidence indicating that these cells perform better *in vivo* in animal models or patients.

FUNCTIONAL VALIDATION OF RPE 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 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, confluency and pigmentation (8); expression of RPE markers (12, 71); barrier or trans-epithelial resistance (12, 72); functional intracellular calcium signaling (72); polarized cytokine secretion (9, 12); ability to phagocytose POS (9, 12); ability to maintain ionic composition in the sub-retinal space (12, 72); and the ability to transport water from the apical to basal sides (Table 1). It is, however, essential to note that most of the these RPE functions are dependent on an intact monolayer making it harder to validate RPE cell suspension as a clinical product functionally.

Measurements of global 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. 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 (72). Purinergic signaling is critical for *in vivo* RPE functions;

light stimulated ATP released by the photoreceptors causes the activation of purinergic receptors on RPE apical surface. This leads to calcium release from intracellular stores, driving the activation of basolateral chloride channels and fluid flow from the sub-retinal space (apical) into the choroid (basal) (72, 73). 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 (73, 74).

In another example, Sharma et al. 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 as compared to the clone-to-clone variability in different iPSC-RPE manufacturing runs (12). 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 RPE TRANSPLANTS:

There are five main safety concerns for any cell therapy product: **(1)** the presence of infectious agents – bacteria, fungus, mycoplasma, and virus; **(2)** the presence of foreign substances (endotoxin and animal products); **(3)** the presence of contaminating PSCs; **(4)** the presence cells with undesired cell lineages; and **(5)** the generation of cell therapy products which are genomically unstable.

1. Sterility of cell therapy product and all its animal origin reagents is monitored at key banking/cryopreservation stages and in the final formulation as per the 21 CFR section 610.12 guidance document from the FDA (https://www.ecfr.gov/). 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 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.

2. 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 21 CFR section 610.13 (b), the acceptable endotoxin limit is 5EU/kg body weight/dose. It is expected that all these clinical programs have validated their manufacturing process to meet the FDA acceptance limit for endotoxins.

3. 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 teratoma when injected as pure cells subcutaneously in mice (76). This amounts to 0.5% of a clinical dose of 100,000 cells that are currently being used in most RPE transplants. In comparison, Schwartz et al. demonstrated that even 1% ESCs mixed with RPE cells in immunocompromised mice do not lead to any teratoma formation in the sub-retinal space (Table 2, (5, 6). This observation 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%, 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.

4. Non-RPE cells and non-PSCs that may contaminate the RPE transplant or RPE cells that change phenotype after transplantation both can 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 epithelial to mesenchymal transition in transplanted cells, causing cells to lose RPE phenotype, become fibroblastic and form membranous scars that can lead to severe vision loss. Such micro-membranes were detected in some patients that received RPE cells in suspension in the Schwartz et al. study (5, 6) (Table 4). 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.

5. Two landmark publications have recently highlighted the issue of PSC genomic instability. Merkel et al. showed enrichment of p53 mutations in ESCs or iPSCs cultured for more extended periods (77). Although most cell lines tested in this study were generated under non-GMP environment, this observation is supported by previous work showing that cells in culture tend to accumulate genomic alterations that provide survival advantage (78, 79). Mandai et al. reported copy number variations in clinical-grade iPSCs reprogramed from AMD patient's skin fibroblasts (10). Genomic alterations can be acquired in iPSCs during reprogramming process or can be enriched from pre-existing 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 (Table 1, (12). This may be since CD34+ cells are progenitor in nature and continue to retain their proliferative potential when expanded *in vitro* (81). Furthermore, as demonstrated by Sharma et al.,

oncogene exome sequencing, along with standard G-band karyotyping, may provide relevant information to encourage the safety of cell therapies derived from iPSCs.

Most preclinical studies performed to date have tested tumorigenic, toxicity, and migratory profile of PSC-derived RPE cells, in addition to confirming their sterility and lacking endotoxins (5, 6, 8–12). Immuno-compromised 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 design 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, whereas, for autologous cell therapy products, only cells made from 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 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 safe profile of their respective RPE transplant (5, 6, 8–12).

EFFICACY OF RPE 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 disease pathology of AMD and allow testing RPE transplants as replacement tissue. Currently, no such model exists. Most efficacy studies have used the Royal College of Surgeon (RCS) rat model where dysfunctional RPE cells stimulate retinal degeneration. RCS RPE cells cannot phagocytose photoreceptor outer segments (POS) due to a mutation in a gene – MERTK, involved in outer segment phagocytosis (8, 82, 83). One of the caveats with RCS rat 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 non-specific 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 (Tables 2, 3 (8–10, 12, 87). 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. published a laser-induced RPE injury in a pig model that displays photoreceptor degeneration triggered by laser induced RPE-cell death and thus recapitulates some features of AMD pathology, (12). A 2×4 mm human iPSC-RPE patch is transplanted into a laser-injured pig eye before the photoreceptors degenerate, and the RPE patch integrates into

the back of the eye within ten weeks, begins phagocytosing the POS, and limits 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 direct drug properties, but they indirectly do provide a therapeutic benefit (Figure 1 and Tables 1 and 3). The scaffold aides in RPE monolayer polarization and its delivery as a patch. Furthermore, 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-co-glycolic) 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 ECM and form a Bruch's membrane-equivalent structure (12, 88). Over time, the PLGA completely degrades, allowing the RPE-patch to integrate with the pig eye. It is not sure 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, the method of RPE differentiation, and the clinical dose used, the surgical tool used in these studies is another critical variable (5, 6, 8–12). Table 3 summarizes various approaches used for 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 that in many cases requires folding a large RPE patch 6×3 mm in size and unrolling it in the sub-retinal space (8), or 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 on end of the patch to roll it before transplantation (8). In any case, the patch transplant procedure is surgically more invasive, may require longer recovery times, and often also result 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 have been tested in twenty-nine patients, and only one patient has been transplanted so far 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 twenty-two patients received RPE suspension (11 AMD and 11 STGD). Although this patient population is too small to make a comparative analysis of the clinical data collected from these six clinical studies, the safety of RPE transplant is confirmed by all of these studies. No severe adverse effects were seen in any of the patients. Most of the adverse effects were either related to the immunosuppression regimen or the dosing procedure (Table 4).

The target population consisted of mostly advanced AMD patients, fifteen dry AMD patients, eleven STGD patients, and three wet AMD patients (Table 4) (5, 6, 8–10). 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 solely attributed to the patch, or if the surgery itself also provided some benefit. Previous work suggests that a sub-retinal 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 RPE-transplant potency. As some of these trials advance to commercial stages, the mechanistic insights into the potency of RPE-transplant 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 at early stages while the patient population targeted 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 at phase 2 and 3 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, the manufacturing process and sub-retinal delivery of the transplant may be one of the factors in its long-term integration and survival. Phase 2 and 3 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 revival of 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 approval of such advanced cell therapy products is in sight now.

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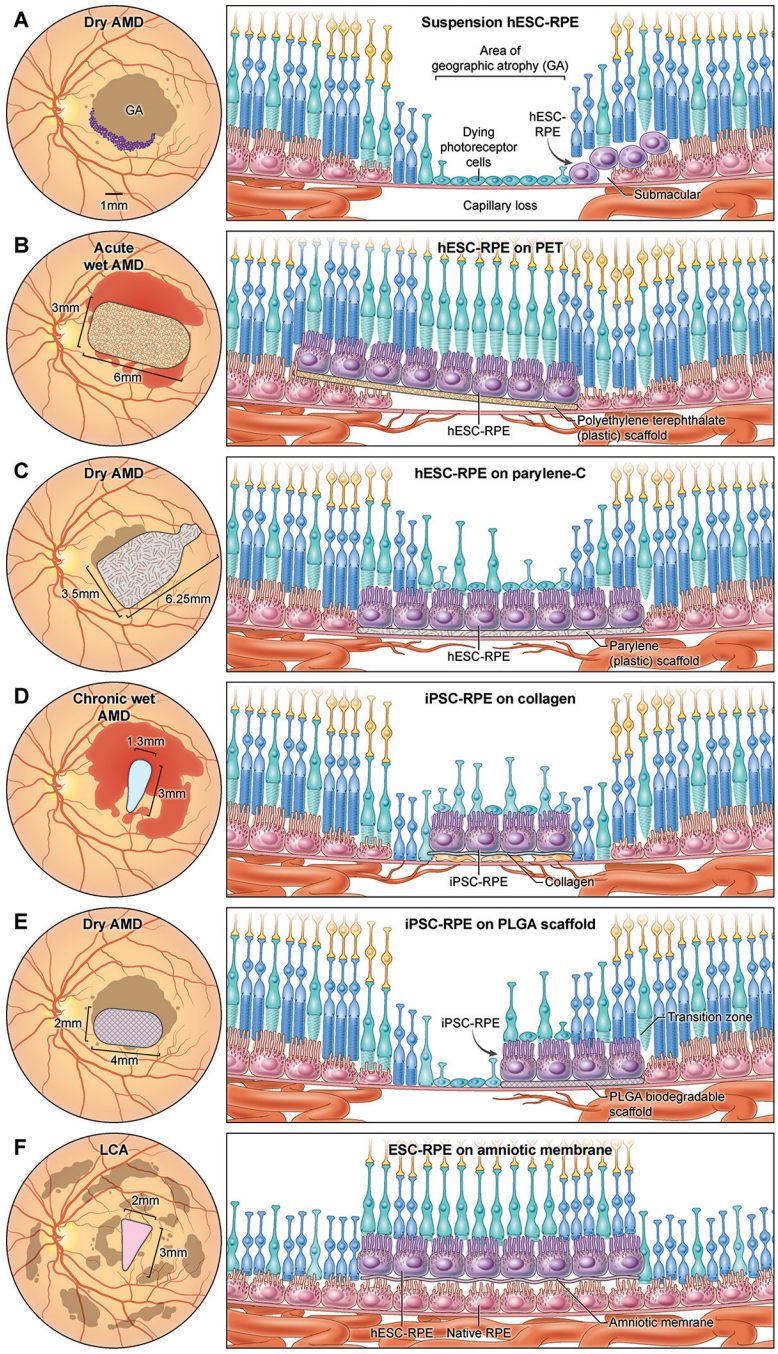

FIGURE 1.

Schematic of various ongoing and planned RPE-transplant approaches. Left panels show a fundus view of the transplant. Right panels show how transplants (purple) would be integrated into the sub-retinal space, their possible impact on retina and choroid and the various scaffold materials. **(A)** Schwartz et al. injected ESC-RPE cell suspension in the sub-macular region. ESC-RPE cells in suspension do not form a polarized monolayer but stay as a bolus of rounded non-polarized cells in the sub-macular region (5). **(B)** Da Cruz et al. 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 rescue photoreceptors that may not have degenerated in these specific patients (8). **(C)** Kashani et al. used an ESC-RPE patch on a parylene scaffold, transplanted in the area of geographic atrophy. 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 (9). **(D)** Mandai et al. 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 (10). **(E)** Sharma et al. propose to transplant autologous iPSC-RPE patch at the border of geographic atrophy 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 GA lesion (12). **(F)** M’Barek et al. propose to test a gelatin embedded ESC-RPE patch 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.

Table 1:

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

Author	PSC source	PSC-RPE differentiation method	Transplant functional validation
Schwartz et al., 2012; **2015a; 2015b**; Song et al., 2015	ESC (MA09), allogeneic	**Spontaneous (timeline NA)** **1** ESC grown on mouse feeder **2** RPE at passage 2 were used for transplantation	**RPE validated before purification and banking** **1** IF and q-RT PCR for PAX6, ZO1, BEST1, MITF **2** Phago: flow cytometry based for phRODO particles **3** Melanin content measurement spectrophotometrically **4** lacking OCT-4 ICC and Alkaline Phosphatase staining
Mandai et al., 2017 Osakada et al; 2009	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 validated** **1** Purity by BEST1/PAX6 **2** >95% by immunostaining **3** TER (290 Ohms.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., 2018; **Lu et al., 2012; Pennington et al., 2014;**	ESC (H9), allogeneic	**Spontaneous differentiation (32 weeks)** **1** 16 weeks of spontaneous differentiation in on Synthemax coated plates. **2** Pigmented patches manually picked and dissociated using TrypLE and seeded on VTN coated plates **3** passaged three 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., 2018	ESC (SHEF01), allogeneic	**Spontaneous differentiation (30–58 weeks)** **1** up to 22 weeks of spontaneous differentiation on Vitronectin coated plates. **2** Pigmented patches manually picked and expanded on CELLstart coated plates for 5–16 weeks	**PET-RPE patch validation** **1** Visual inspection **2** Loss of Lin28 **3** PMEL17 ICC **4** Lacking Lin28 expression

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Author	PSC source	PSC-RPE differentiation method	Transplant functional validation
		3 RPE cells matured on PET membrane for 3–20 weeks days	**5** PEDF secretion **6** Phagocytosis of labeled POS by IF
M'barek et al., 2017	ESC (RC-9), allogeneic	**Spontaneous differentiation (17 weeks)** ES line derived on fibroblast, later made feeder free using CELLstart 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
Sharma et al., 2019	Autologous iPSCs	**Directed differentiation (monolayer culture; 11 weeks)** **1** iPSC grown on VTN **2** iPSCs induced to optic neuroectoderm using LDN-193189, SB431452, CK1–7, PD0325901, IGF-1 (12 days) **3** Optic neuroectoderm induced to committed RPE using Activin and Nicotinamide (10 days) **4** Committed RPE growth phase (22 days) **5** enrichment of RPE progenitors using CD24 and CD56 antibodies and maturation on PLGA scaffolds (5 weeks)	**PLGA-iRPE patch validation** **1** Purity by flow for PMEL17, TYRP1, BEST1, CRALBP **2** lacking OCT4, SSEA4, TRA1–81by flow cytometry **3** SEM, TEM **4** IF for RPE65, GPNMB, COLIV, and COLVIII **5** qRT-PCR for RPE65, BEST1, GPNMB, MYRIP, TYRP1, ALDH1A3, TRPM1, OCA2 **6** Shape Metrics **7** TER (>400 Ohms.cm^2) **8** VEGF secretion **9** POS phagocytosis

Abbrev: ESCs – embryonic stem cells, FGF2 – fibroblast growth factor 2, ICC – immunocytochemistry, IF – immunofluorescence, IGF-1 – insulin-like growth factor -1, iPSCs – induced pluripotent stem cells, PEDF – pigment epithelium derived factor, PET – polyethylene terephthalate, PLGA – poly-(lactic-co-glycolic) acid, POS – photoreceptor outer segments, PSCs – pluripotent stem cells, SEM – scanning electron microscopy, TEM – transmission electron microscopy, TER – trans-epithelial resistance, VEGF – vascular endothelial growth factor, VTN – vitronectin, RPE-retinal pigment epithelium, qRT-PCR-quantitative real time-polymerase chain reaction, ROS-rod outer segment.

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Table 2:

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

Author	Animal model	Study purpose, dose	Study conclusion
Schwartz et al., 2012	NIH-III immune-compromised mice	Tumorigenicity and tissue distribution **1** 50,000 and 100,000 hESC-RPE **2** Spiking of 0.01%, 0.1% and 1% ESCs in 100,000 ESC-RPE	**1** No human origin tumor detected **2** No Ki67 positivity detected in hESC-RPE **3** No cells found outside the eye
Da Cruz et al; 2018	**1** NIH III immune-compromised mice **2** Healthy pigs	**1** Tumorigenicity, 6×10^4 hESC-RPE **2** Feasibility, tissue distribution, safety, 3 × 6 mm RPE-patch	**1** No human origin tumor detected **2** No proliferation, No migration **3** Photoreceptor survival over RPE-patch
Kashani et al., 2018; Koss et al.,2016	Healthy pigs	Feasibility and safety, 3.5 × 6mm RPE-patch	**1** None of the animals had high IOP. **2** 75% pigs with good implant placement **3** No inflammation or migration of RPE cells
Mandai et al., 2017; Kamao et al 2014; Kanemura et al., 2014	**1** Healthy cynomolgus monkey **2** NOG-SCID mice, **3** Nude rats,	**1** survivability and safety, iPSC-RPE patch 1×2 mm **2** tumorigenicity, subcutaneously transplants, 1×106 cells **3** tumorigenicity, sub-retinal transplants, 1×1 mm patch	**1** No human origin tumor detected **2** Graft rejection in allotransplants, **3** Autotransplants survived for 12 months **4** Dislodged RPE cells noted in vitreous
Sharma et al., 2019	**1** Nude rats **2** Pigs with laser-injured RPE	**1** Tumorigenicity and toxicity -iPSC-RPE patch ($0.5mm^2$) -iPSC-RPE suspension (100,000 cells) **2** -Feasibility and efficacy -iPSC-RPE sheet (2×4 mm) -iPSC-RPE suspension (100,000 cells)	**1** No human origin tumor or teratoma **2** No systemic toxicity **1** iPSC-RPE patch integration **2** No degeneration of PRs over sheet **3** Human RPE phagocytosing pig POS
M' Barek et al.,2018	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

Abbrev: ESC-RPE- embryonic stem cells-retinal pigment epithelium, IOP- intra ocular pressure, iPSC-RPE- induced pluripotent stem cells-retinal pigment epithelium, POS-photoreceptor outer segments.

Table 3:

Comparative assessement of transplantation devices used for delivery of RPE transplants

Manuscript	**Schwartz et al., 2012; 2015**	**Da Cruz et al., 2018**	**Kashani et al., 2012 (Fernandes et al., 2017)**	**Mandai et al., 2017**	**Sharma et al., 2019**	**M'barek et al., 2017**
Device Name	MedOne PolyTip® Cannula	Introducer tool	Tissue injector	Customsurgical device	Trasnplantation tool	Tissue injector
Type of Transplant	Cell suspension	Rolled RPE-patch	Rolled RPE-patch	Flat RPE-patch (no scaffold)	Flat RPE-patch	Rolled RPE-patch (gelatin embedded)
Cannula size	25 G	Not avaiable	17 G	20 G modified intravenous cannula	2.5 mm	Not avaiable
Implant Size (dosage)	150 uL Cohort 1: 50,000 cells Cohort 2: 100,000 cells Cohort 3: 150,000 cells	3 × 6 mm PET scaffold (~100K cells/patch)	3.5 × 6.25 mm Parylene scaffold (~125K cells)	1.3 × 3 mm no scaffold (4,500 < viable cells/mm^2)	2 × 4 mm PLGA scaffold (~75K cells/patch)	2 × 3 mm hAM scaffold (~50K cells/patch)
Tool operation	VFI	Manual, rotary control	Manual, push button	Manual, plunger	VFI	Manual, plunger

Abbrev: VFI – Viscous Fluid Injector, RPE-Retinal Pigment Epithelium, PET- polyethylene terephthalate, PLGA- poly-(lactic-co-glycolic) acid, hAM-human amniotic membrane, G-gauge.

Table 4:

Summary of patient outcomes for ongoing and planned pluripotent stem cell-derived RPE transplant studies

Author	Patient population	Outcomes	Ocular adverse events
Schwartz et al., 2012; **2015a; 2015b**; Song et al., 2015	Dry AMD: 11 patients (70–88 years) BCVA 20/200-hand motion STGD: 11 patients (20–71 years)	**Median follow up 22 months** **1** AMD: median VA increase treated vs untreated eye (14 letters vs 1 letter, p 0.0117). **2** STGD: Trend toward improved VA in treated eye (12 letters). No adverse proliferation	**1** One eye – endophthalmitis **2** One eye – vitreous inflammation that got resolved by 6 months **3** Three eyes – preretinal patches, non-contractile **4** Four eyes - cataract
Mandai et al., 2017	One wet AMD (77 year) patient, BCVA 20/200 right eye	**1 year follow up** **1** No evidence of leakage or recurrence of hemorrhage **2** No need for additional anti-VEGF injections **3** BCVA maintained at 20/200 **4** Fixation shifted closer to the transplant	**1** Choroidal hemorrhage 3 days post transplantation **2** Scaffold curling **3** Cystoid macular edema
Kashani et al., 2018	Dry AMD: 5 patients (69–85 years) Visual acuity on ETDRS chart (range 3–22)	**4–12 months follow up** **1** Three patients VA stable **2** One patient showed 17 letter improvement **3** All 4 patients had ELM detectable by OCT over the area of the transplant **4** Three patients – fixation detected over the transplant	**1** One patient could not be transplanted **2** Mild to moderate surgery associated hemorrhage was noted in all 4 patients. It resolved in 3 without intervention. One required anti-VEGF injections.
Da Cruz et al., 2018	Acute wet AMD: 2 patients (60 and 84 years) Visual acuity on ETDRS chart (10 and 8)	**4–12 months follow up** **1** Patient 1 −19 and patient 2 −21 letters improvement **2** RPE cell migration off the patch **3** Fixation changed to the area of the patch	**1** Exposure of the suture for fluocinolone implant in patient 1 **2** Worsening of diabetes in patient 2 **3** PVR with tractional membranes in patient 2

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