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Differentiation of Pluripotent Stem Cells into Retinal Pigmented Epithelium

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Abstract

Ocular diseases affect millions worldwide and dramatically influence the quality of life. Although much is known about ocular biology and disease pathologies, effective treatments are still lacking. The eye is well suited for application of emerging cell-based therapies. This chapter explores the development of stem cell-based treatments for age-related macular degeneration (AMD), a prevalent ocular disease in the elderly. Retinal pigmented epithelium (RPE), a cell type implicated in AMD, has been derived from both induced pluripotent stem cells and embryonic stem cells (ESC). Rapidly advancing research has generated various methods of RPE differentiation and several transplantation strategies. Clinical trials are already underway using suspensions of ESC-derived RPE and others are soon to follow. This chapter will provide an overview of current derivation and transplantation strategies for stem cell-derived RPE for the treatment of AMD and other related ocular diseases.

Age-related macular degeneration (AMD) is a progressive disease leading to the loss of high-acuity vision. More than 200,000 people are diagnosed per year, making AMD the leading cause of blindness in the elderly in the developing world [1]. Around 7.2 million people in the US alone suffer from this devastating disease with annual medical costs exceeding USD 250 billion [2]. The disease primarily affects a small region in the back of the eye responsible for central vision called the macula. Loss or dysfunction of macular retinal pigmented epithelium (RPE), which provides crucial supportive functions for the photoreceptors, is thought to play a crucial role in disease progression. There are two general forms of the disease, wet (exudative) AMD and dry (nonexudative) AMD, with over 90% of patients suffering from the more slowly progressing dry form [3, 4]. Wet AMD is characterized by a quick progression of choroidal neovascularization (CNV), leading to rapid degradation of RPE and photoreceptors and impaired vision. However, this form of the disease is successfully treated in most patients using inhibitors of the vascular endothelial growth factor, such as ranibizumab, bevacizumab, and aflibercept [1, 5–7]. The advanced form of AMD not involving CNV growth, termed geographic atrophy, results in extensive RPE and photoreceptor death, which causes substantial vision loss as the fovea becomes threatened or involved di-

rectly [8, 9]. This extensive loss of vision impairs daily activities including reading, driving, face recognition, and mobility. Currently, there are no reliable, effective treatments for dry AMD [10, 11]. Although some experimental autologous transplants and retinal translocation surgeries have been performed, they are fraught with obstacles and complications that have yet to be overcome [12].

AMD is a multifactorial disease with numerous genetic and environmental risk factors [13, 14]. Polymorphisms within multiple complement system genes, including alternate complement factors H, B, and I, in addition to components 2 and 3, lead to substantial genetic predisposition to AMD [15–21]. These findings suggest a chronic inflammatory state in the disease and potentially a progressive immune attack on RPE. Mutations in noninflammatory-related genes, apolipoprotein E *(APOE)* [22], hepatic triglyceride lipase *(HL)* [23], cholesteryl ester transfer protein *(CETP)* [23], vascular endothelial growth factor *(VEGF)* [24], and regions surrounding the tissue inhibitor metalloproteinase 3 *(TIMP3)* [23], also correlate with AMD onset. Genes associated with AMD also include Age-related Maculopathy Susceptibility 2 *(ARMS2)* [21] and HTR1A serine peptidase 1 *(HTRA1)* [25], although little is known about their mechanisms of action. A mutation in the ATP-binding cassette transporter gene sub-family A (ABCA4) leads to Stargardt disease, causing early-onset macular degeneration, often rendering patients blind before the age of 20 [26]. ABCA4 mutations may also be associated with some cases of AMD [27]. Environmental factors that influence AMD development and progression include, age, smoking, non-African descent, poor eating habits, and low physical activity [14]. The precise mechanism of AMD causation is still not completely understood.

The presence of drusen deposits is the hallmark pathology of both forms of AMD. Drusen are composed of lipids, carbohydrates, proteins, and cellular components, including secreted inflammatory proteins [28, 29]. These deposits build up in between the basal laminar side of the RPE and the surface of the Bruch's membrane, a multilaminar extracellular matrix (ECM) barrier separating the RPE and choroid. The monolayer of RPE is disrupted by drusen and may play a role in RPE dysfunction. However, the direct effect of drusen on RPE and in AMD is still unclear [28]. Drusen biogenesis might cause RPE dysfunction, or RPE damage could lead to defective degradation of cellular components and consequent formation of deposits [30]. Regardless, drusen are present in AMD where RPE undergo apoptosis, contributing to photoreceptor death [31, 32].

RPE are critical to photoreceptor viability [33, 34]. The RPE create the blood-retina barrier and have multiple roles in maintaining photoreceptor health and visual function. RPE phagocytose rod outer segments, absorb of stray light, secrete trophic factors, and assist in visual cycle retinol conversion and nutrient diffusion from the choroid. Therefore, dysfunctional RPE leads to the subsequent damage and death of photoreceptors. Photoreceptor loss within the macula causes central vision impairment with disease progression expanding the extent of vision loss.

Retinitis pigmentosa (RP), Leber congenital amaurosis and Best disease are also associated with RPE dysfunction and might benefit from novel cell replacement therapies. Current treatments, in various stages of development, involve gene therapy using associated adenoviral injections [35, 36]. Promising research using merTK gene therapy in mouse models of retinal dysfunction could lead to a novel RP treatment; merTK is a receptor located on the apical side of RPE, critical in phagocytosis of photoreceptor outer segments. RPE65 gene therapy is currently in clinical trials for Leber congenital amaurosis treatment. RPE65 is an RPE-specific enzyme involved in the retinoid visual cycle, necessary for continual photoreceptor function [36]. To gain a better mechanistic understanding of these diseases, induced pluripotent stem cell (iPSC) lines are being gener-

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Dev Ophthalmol. Basel, Karger, 2014, vol 53, pp 81–96 (DOI: 10.1159/000357361)

ated from patients. The Gamm group has created several iPSC-derived RPE (iPSC-RPE) lines from Best disease patients to elucidate the normal and aberrant function and localization of bestrophin, the protein genetically altered in this disease [37].

The Case for Cellular Therapies

Current available treatment options for dry AMD are limited. Vitamin and mineral therapy can reduce the incidence of CNV but does not seem to affect the development of GA [38]. Although cell-based therapies have challenges, including cellular production and characterization, immune rejection, inflammatory response, tumor formation, and integration and survival of the transplant, the eye has many advantages that make this approach feasible. The macula averages 6 mm in diameter, requiring approximately 120,000 RPE cells for complete coverage. The subretinal space is immune privileged, lacking significant immune cell infiltration. The eye is also accessible, and refined surgical techniques and tools are available for efficient subretinal transplantation. In addition, good end point parameters of transplant success can be measured. Visual acuity can be determined using simple tests (EDTRS Eye Charts) as well as more complex microperimetry. Advanced imaging techniques, such as optical coherence tomography and fundus autofluorescence can be done to assess transplant placement, RPE integrity, photoreceptor integrity, RPE-photoreceptor integration, choroidal thickness, and complications such as retinal edema and CNV. Progress in imaging is advancing at a rapid pace, with the development of novel noninvasive adaptive optic methods that can resolve single RPE and photoreceptor cells [39].

Proof of concept for RPE transplantation comes from studies in rodents and humans that began 20 years ago. Extensive research has been carried out using a variety of cell types, transplantation methods and tools, and retinal dysfunction models [12]. In rodents, while there is no ideal model for AMD, the Royal College of Surgeons (RCS) rat is used as a model of RPE dysfunction. It is important to note that rats do not have a macula (including a cone-rich fovea), and the RCS rat model does not recapitulate some critical aspects of human AMD (e.g abnormalities in Bruch's membrane structure and composition). RCS rats harbor mutations in the merTK gene, which encodes a cell surface receptor required for the critical RPE process of photoreceptor outer segment phagocytosis. Without proper phagocytosis, RPE fail to maintain photoreceptor viability, causing substantial vision loss 3 months after birth. A number of cell types will rescue vision in this model. Suspensions of transformed and spontaneously derived RPE cells, h1RPE7 and ARPE19, injected into the subretinal space show improved visual performance and histology compared to sham-treated RCS rats [40–43]. Isolated fetal human neural cortical precursor cells also rescue visual function after injection. These precursors migrate to the retina and form a multi-layered structure on the Bruch's membrane, increasing retinal sensitivity and function [44]. Although fibroblasts will not effectively rescue vision in the RCS rat, some salutary effects are observed in animals undergoing subretinal injection without cell delivery [45]. In addition, the photoreceptor rescue effect often extends hundreds of microns beyond the border of the transplanted cells [43]. Therefore, it seems likely that secretion of trophic factors and activation of endogenous macrophages that clean up the debris zone contribute to the rescue and preservation of photoreceptors.

To date, human RPE transplantation has utilized three types of RPE: autologous, allogenic, and stem cell-derived. Autologous RPE transplantation involves excision of a patch of healthy RPE plus choroid from the ocular periphery followed by placement under the macula. Macular translocation, another form of autologous surgery, originally used to treat exudative AMD patients, involves retinal detachment and rotation,

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locating healthy RPE and Bruch's membrane under the macula. However, when macular translocation is done to treat GA, GA develops rather rapidly under the new location of the fovea for unknown reasons [46]. Fetal and cadaver RPE have been injected in suspension, small sheets, or gelatin matrices into patients with varying ocular diseases to improve visual performance [12]. There is a suggestion that better results have been obtained with patches or sheets, rather than suspensions of cells [47, 48]. However, the results of RPE transplantation studies in AMD patients have been variable and time will tell which method proves to be superior [49]. The relatively few documented cases of visual improvement following RPE transplantation demonstrate that cellular therapy is feasible and may be a useful approach for treating selected AMD patients.

A number of important issues must be addressed for cell-based AMD therapy to be optimized, e.g. long-term cell survival (independent of immune rejection), identification of the stage(s) of the disease most appropriate for surgical intervention, and development of a safe, simple surgical technique. A critically important issue also involves identification of sources of cells for transplantation. Apart from ethical concerns, fetal RPE (fRPE) are not available in abundance and cannot be propagated indefinitely (abnormal morphology is evident by the fifth passage in most cases), which limits their commercial utility. The ideal cell source for therapies would have infinite expansion properties, reproducible and well-defined differentiation capabilities, and stable cryopreservation and shipment abilities. Human pluripotent stem cells meet the starting cell material criteria for the large source of RPE needed for ocular disease cell therapy.

In this chapter, we will discuss the methods of RPE derivation from pluripotent stem cells and the various transplantation methods undergoing development as cell therapies move forward into the clinics. While many excellent reviews have been written on this topic [47, 50–52], the field is developing fast enough to warrant a new discussion.

Derivation of Retinal Pigmented Epithelium from Pluripotent Stem Cells

There are two main types of human pluripotent stem cells: human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs). hESCs are harvested from the inner cell mass of a 3- to 5-day-old fertilized embryo. hESCs can be cultured indefinitely with maintained stem cell characteristics and the potential to differentiate into all three germ layers [53]. The first five hESC lines generated in 1998 by Prof. Jamie Thomson's laboratory at the University of Wisconsin are still in use today. Theoretically, these lines could be used to supply enough cells for research and treatment of all patients of any disease for which cellular therapies are developed, including AMD. iPSCs are derived from differentiated somatic cells that have been reprogrammed to revert back to a pluripotent state. This reprogramming was first accomplished using integrative viral vectors encoding specific embryonic transcription factors associated with pluripotency. The stem cell colonies that formed, like hESCs, could divide infinitely and differentiate into any cell type in the body [54, 55]. The potential advantage of iPSCs over hESCs is that patient-specific cells could be generated that might avoid immune rejection after autologous transplantation. While initial reports suggested that autologous undifferentiated iPSCs might still be rejected [56], more recent studies have shown that differentiated cells are immune compatible [57, 58]. Improved methods for generation of iPSCs that lack integrating vectors have been discovered that utilize excisable transgenic systems, micro RNAs, modified mRNAs, and small molecules [59–63]. Thus, both iPSCs and hESCs are promising sources for future cellular therapies.

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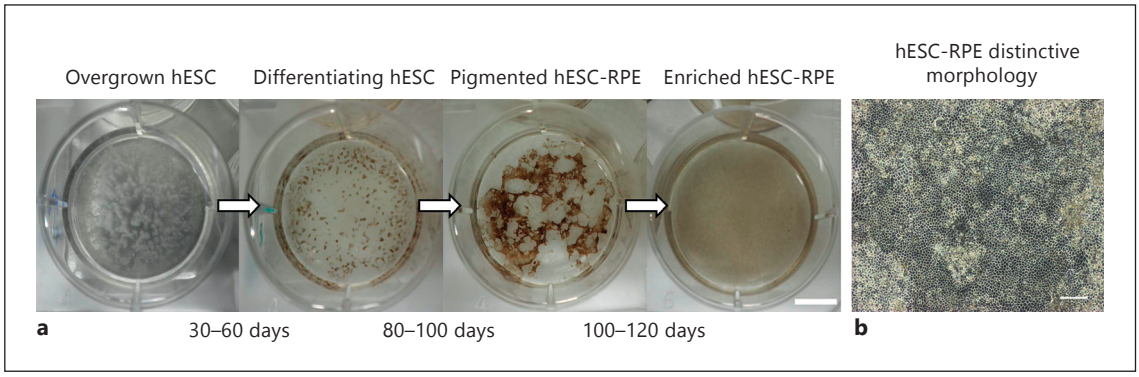

Fig. 1. Depiction of spontaneous RPE differentiation from hESCs using a continuous adherent culture method. From left to right, overgrown hESCs through the spontaneous differentiation process. **a** Pigmented colonies are excised after 100 days in culture and re-plated to yield a mostly homologous population of RPE cells. **b** Enriched RPE exhibit typical cobblestone cuboidal morphology and pigmentation. Scale bar = 10 mm (**a**) and 100 μm (**b**).

Numerous cell types have been derived from both hESCs and iPSCs including RPE. RPE differentiation from hESCs was first described in 2004 by Klimanskaya's group, and since then multiple groups have optimized the protocol for producing a pure population of hESC-derived RPE (hESC-RPE) and iPSC-RPE [40, 47, 50, 64–74]. In culture, stem cells and RPE need to be grown on a substrate to support their attachment and growth. hESCs and iPSCs originally were grown on inactivated mouse or human embryonic fibroblasts; however, Matrigel, a composite of ECM proteins from a mouse sarcoma, and mouse PA6 stromal cells have also been used [53–55, 64, 75]. Endogenous RPE normally reside on an ECM layer called Bruch's membrane. In culture, a similar substrate is needed to facilitate RPE attachment, differentiation, and survival. Several substrates have been used: human Bruch's membrane explants, gelatin, laminin, vitronectin, bovine corneal endothelial cell ECM, and Matrigel. Comparisons show that Matrigel, laminin, vitronectin, and bovine corneal endothelial cell ECM lead to the purest RPE phenotype [76–78]. Ideally, a xeno-free (non-animal containing) protocol would be used throughout the entire differentiation process.

The Continuous Adherent Culture Method

Derivation of RPE from either pluripotent starting source can be achieved in a continuous adherent culture. Stem cells are allowed to overgrow on a feeder cell layer of mitotically inactivated mouse embryonic fibroblasts or Matrigel. Stem cell colonies become confluent and multilayered. They lose their tight borders and begin to differentiate. At this time, the media is changed from a stem cell-supporting media, to a differentiating media without basic fibroblast growth factor (bFGF) to further promote differentiation. Cultures maintained in this bFGF-free media will begin to show small spots of pigmentation after 1–8 weeks. These spots are differentiating RPE. Over time, the pigmented spots multiply and expand within the culture. Although there are other differentiated cell types in the cultures, the spontaneously derived RPE are easy to distinguish because of their pigment granules, a major advantage in hESC/iPSC-RPE differentiation. When the pigmented RPE spots become frequent and large, they can be mechanically dissected and passaged [40, 65–67, 73, 79, 80]. The excised cells are grown to a monolayer and exhibit the RPE-distinctive cobblestone morphology and pigmentation patterns (fig. 1).

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The downside of this RPE enrichment method is that the manual isolation technique is difficult to fit within the Good Manufacturing Practice (GMP) production guidelines. This limitation could prevent advancing hESC/iPSC-RPE cellular therapies into human clinical trials. Therefore, enzymatic techniques are under investigation to separate RPE from other differentiated cell types [80].

After the pigmented RPE are enriched and plated, quality control must be performed to ensure these derived cells express similar gene expression patterns and function as endogenous RPE. Assays to establish RPE identity and potency must be created. Quantitative real-time PCR can be done to compare transcript levels of hESC and iPSC-RPE with human RPE. A number of RPE genes [e.g RPE-specific transcription factors, microphthalmia-associated transcription factor (MITF) and orthodenticle 2 isoform b homeobox protein (OTX2); visual cycle proteins, cellular retinaldehyde-binding protein (CRALBP) and RPE protein 65 kDa (RPE65); secreted factors, pigment epithelium-derived factor (PEDF); tight junction marker, zona occludens 1 (ZO-1), and phagocytosis component, mer tyrosine kinase (MERTK)] are analyzed by quantitative real-time PCR to ensure mRNA levels coincide with human fRPE [40, 65–67, 73, 79]. Current data suggest some differences between fRPE, the benchmark comparison. Overall, bioinformatics analyses show that hESC-RPE are very similar to fRPE but may correspond to a less mature RPE state [73]. RPE65, a mature RPE marker, appears to increase with culture of hESC-RPE, and could also be used as a biomarker to determine RPE identity. It is interesting to note that fRPE share a much more similar gene profile with hESC-RPE than iPSC-RPE [73]. In addition, hESC-RPE and fRPE show equivalent low levels of genes associated with aging, energy metabolism, and the complement system, all factors associated with AMD pathophysiology [73; Hikita et al. unpubl.].

Stem cell-derived RPE must also be tested to ensure these cells carry out critical physiological processes. As described previously, RPE are responsible for phagocytosing photoreceptor outer segments to maintain photoreceptor homeostasis. Phagocytosis can be tested using an assay in which fluorescently labeled outer segments are incubated with cultured RPE, and the amount of internalized segments is quantified. hESC-RPE and several iPSC-RPE lines have phagocytic abilities comparable to fRPE [66, 67, 73, 76, 79]. Several studies have implanted both types of stem cell-derived RPE into rodent models of retinal dysfunction and shown improved visual performance [40, 65, 68, 81]. In addition, subretinally injected iPSC-RPE enhanced visual acuity in a model of RP with no tumor formation [82]. Currently, all clinical trials involving hESC-RPE require immunosuppression, which could potentially be avoided if a well-defined, rapidly developed iPSC-RPE line could be generated. One example of such an approach uses only Oct4 to reprogram into iPSCs in combination with small molecules [83]. These lines have been derived into RPE and hold great potential for clinical application of autologous RPE grafts.

The Embryoid Body Approach

hESCs and iPSCs can also be differentiated into RPE using a second strategy called the embryoid body (EB) approach. Although this method may not be as efficient as the continuous adherent approach, it still yields viable RPE and is a spontaneous differentiation protocol [65]. To apply this method, stem cells are grown on feeder cells or substrate until 50–75% confluent. Colonies are dissociated using an enzyme, such as collagenase, and sectioned into pieces. The pieces are plated in a serum- and bFGF-free differentiation media on plates coated with a neutrally charged hydrophilic hydrogel to diminish attachment. The cells will form spherical aggregates called EBs. These bodies can differentiate into all three germ layers and show pigmentation. Twenty days following EB

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formation, the aggregates are passed onto a coated plate, allowing for cell attachment. Within 24 h, cells will attach and begin to spread. Over time, distinct RPE sheets will form. These RPE colonies can be excised manually and passaged onto another coated dish, creating a mostly homogenous RPE cell population [68, 69, 72, 79, 84]. Gene expression profiles of resultant hESC-RPE cells show patterns similar to fRPE, with robust expression of RPE specific genes: MITF, OTX2, CRALBP, RPE65, PEDF, ZO-1, MERTK [68, 69, 72, 74, 84]. In addition, these cells can phagocytose outer segments and rescue visual performance in rodent models of retinal dysfunction [68, 72, 79, 84]. This desired phenotype can also be achieved deriving RPE from iPSCs [69, 71, 85–87].

Directed Differentiation

Spontaneous differentiation of RPE is time consuming, and efficiency varies between cell lines. Therefore, a number of groups have focused on improving protocols to speed up this process. The strategy is to mimic normal human RPE development by adding small molecules to initiate signaling cascades at the correct time to promote retinal cell fate in vitro [69–71, 84]. These efforts have not only focused on hESC and iPSC differentiation into RPE, but also on a range of retinal cell types [88]. RPE are ectoderm and originate from the anterior neural plate neuroepithelium during optic cup development. A range of signaling cascades including the transforming growth factor-β (TGFβ) superfamily, Wingless-related integration site (Wnt), and FGF pathways are involved in RPE specification [33, 89]. Stem cells must first be directed towards a retinal progenitor fate before terminal RPE differentiation. Several groups have directed differentiation by using Wnt and NODAL inhibitors, Dkk-1 and Lefty A, to induce a neural fate [70, 71, 74, 84]. Further neural and retinal induction has been obtained utilizing supplements, N2 or B27 and heparin. Following differentiation into retinal progenitors, RPE can be specified by epithelial driving factors. Activin A, BMP4, Wnt3A and an FGF inhibitor (SU5402) stimulate RPE derivation from neural retinal progenitors [69]. A class B vitamin, nicotinamide, also promotes RPE differentiation, and this effect is enhanced when added in combination with Activin A [72]. Appropriate timing and duration of each molecule is critical to yield pigmented, functional RPE. In 2012, Zahabi et al. [90] reduced the time from beginning human iPSC differentiation to enrichment to 40 days using bFGF, TGFβ receptor inhibitor (SB431542), noggin, retinoic acid, and sonic hedgehog throughout their protocol. A new method of deriving RPE from pluripotent stem cells was published by the Tanaka group in January, 2013. They described the formation of epithelial cysts, mimicking neural tube morphology, arising 5 days after pluripotent cells are embedded in Matrigel. The cysts are extracted, re-plated onto Matrigel-coated wells using an RPE supporting media with supplemental factors, and develop into RPE within 30 days. These RPE become pigmented, express RPE65 and BEST1, show appropriate transepithelial resistance levels, and phagocytose rod outer segments. In addition, following transplantation into the RCS rat, they increase the thickness of the outer nuclear layer within the retina [91]. Most recently, an improved protocol of directed differentiation from hESCs to RPE has been achieved producing enrichable RPE sheets within only 14 days [75] (see fig. 2 for a comparison of all differentiation methods.)

Other Sources of Retinal Pigmented Epithelium

While pluripotent stem cells are the best source of RPE, other stem cell types are under investigation. Both rat neural stem cells, harvested from the cerebral cortex, and mesenchymal stromal

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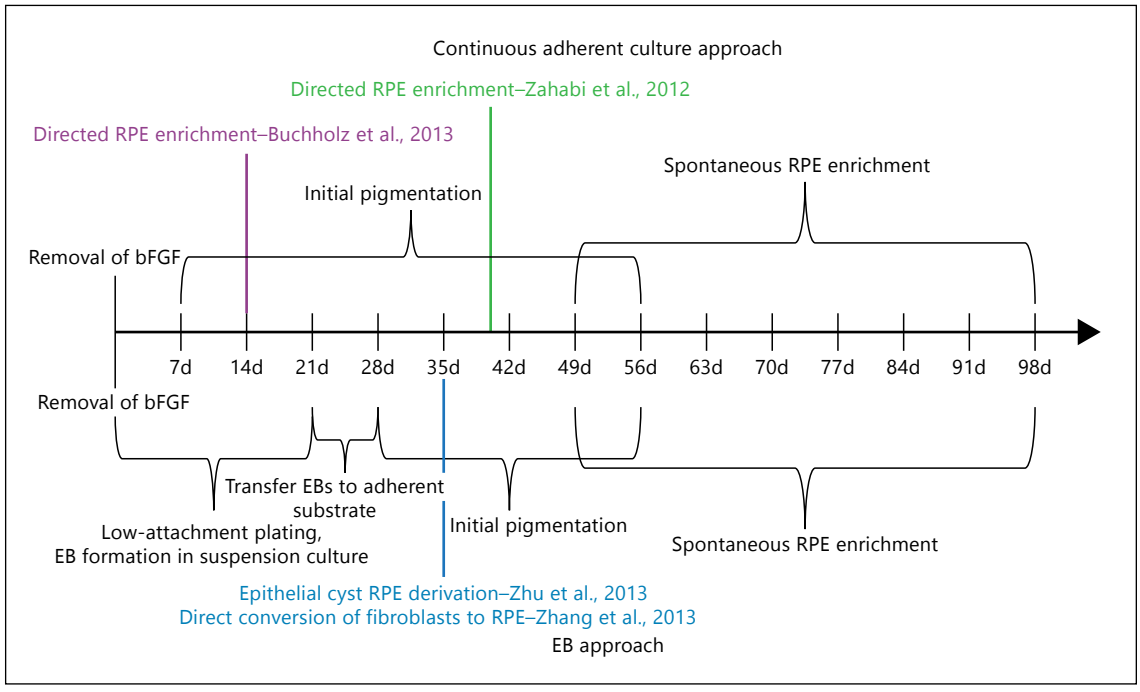

Fig. 2. Time course comparison of the continuous adherent culture and EB culture methods of spontaneous derivation of RPE from pluripotent stem cells. Colored lines indicate time to enrichment for other RPE differentiation methods.

cells (MSC), isolated from human lipo-aspirate, were reported to yield cells with some RPE characteristics using the same differentiation strategy [92, 93]. Co-cultures with rat RPE, or conditioned media from human RPE, in combination with vasointestinal peptide produced cells with some morphological features of RPE [92, 93]. The MSC-derived RPE were further characterized and showed some induction of BEST1 and RPE65, mature human RPE markers [93]. However, the levels of expression were not compared with fRPE, so it is difficult to be sure how RPE-like these cells are. One group used bone marrow-derived mesenchymal stem cells cultured with human RPE-conditioned media and photoreceptor outer segments to generate RPE that express RPE65 and phagocytose porcine rod outer segments [94]. Another potential alternate source of RPE is retinal stem cells (RSCs) extracted from the ciliary margin. Coles et al. [95] reported that there are 10,000 multipotent RSCs in each eye that can yield all retinal cell types, including RPE. After transplantation of RSCs into mice, the cells survived, integrated, and differentiated into the retina. However, a subsequent study questioned whether these were actually RSCs [96]. It remains unclear if these cells can be expanded into a pure RPE population, and more characterization is needed. During development, RPE are plastic cells, and some groups have harnessed this potential in mature RPE, claiming RPE can dedifferentiate into a multipotent stem cell which can then take on other cell phenotypes [97].

A direct conversion of human fibroblasts to RPE-like cells has also been achieved through overexpression of key RPE transcription factors and additional supplements. This protocol yields

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cells expressing some mature RPE markers within 35 days. The function of these RPE-like cells, however, has not yet been reported [98].

Induced Pluripotent or Human Embryonic Stem Cell-Derived Retinal Pigmented Epithelium?

An unanswered question remains: which starting source, hESCs or iPSCs is better for clinical use? A potential drawback to the iPSCs having the same genetic makeup and immunocompatibility as the patient is that they carry the same inherited defects predisposing to the disease. Another caveat is that these cells show increased mutations, copy number variations, and abnormal methylation patterns when compared to hESCs, which could hinder their therapeutic value [86, 99–103]. hESC-RPE are a younger cell type and are not manipulated by viruses or small molecules. Although allogeneic hESC-RPE can be free of disease-associated mutations, they might be rejected by the immune system. The subretinal space is an immune privileged sight, but this privilege is relative and not absolute. Moreover, patients may have a compromised blood-brain barrier, which could allow a larger than normal immune response within the eye. A benefit of hESC-RPE is they can be ready for transplantation immediately following diagnosis in appropriately selected patients. Currently, patient-specific iPSC-RPE would be extremely time-consuming to produce and characterize before transplantation. In patients with rapidly progressing disease, this delay in treatment might result in significant loss of vision. Finally, generation of autologous cells would be costly and require a 9-month tumorigenicity study under current FDA policies. However, banks of HLA-matched iPSCs might overcome this problem.

iPSC-RPE also show memory of their previous terminal fate and show differences in gene profiles from fRPE [104]. These differences, however, vary from cell line to cell line, and with new non-integrative systems, iPSC-RPE appear to be more similar to fRPE [55, 73, 83]. There is great variability between cell lines for both hESC- and iPSC-derived RPE, which has yet to fully be explained and could lead to clinical complications for either source [69, 85, 105, 106]. More hESC-RPE and iPSC-RPE research is needed to elucidate the optimal starting cell material. Therapies moving forward with either cell line must take precautions to ensure reliable and fully characterized cells are manufactured for transplantation.

Transplantation Strategies

In addition to the debate over whether to use hESCs or iPSCs to derive RPE for transplantation, there is also a question about how to deliver the cells. Grafted cells must integrate and function properly with the existing retina to prevent further photoreceptor damage and decrease progression of disease. Two main techniques are being studied currently. The first technique administers a bolus injection of dissociated RPE into the subretinal space. Both hESC-RPE and iPSC-RPE have been delivered with this approach, and both showed photoreceptor rescue and preserved vision in rodent models of retinal dysfunction [40, 65, 72, 81]. An important study in 2009 showed some transplanted cells, in two distinct rat models of ocular disease, survived the duration of the 220-day experiment with no teratoma formation [68]. This study is crucial in proof of principle that cells implanted into the eye can possess the longevity needed to slow disease progression without tumor formation [68]. However, efficiency of cell survival and integration are still in question. Some reports have concluded that the majority of injected cells form aggregates in the subretinal space, do not integrate into the RPE monolayer, and are unaccounted for after a period of time. Importantly, only a subset of injected dis-

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sociated cells possesses the ability to phagocytose rod outer segments [81]. These results are not surprising. It is known that epithelial cells, like RPE, need to maintain contact with a basement membrane to function properly and remain viable [12]. Cells in suspension undergo 'anoikis,' a term coined by Ruoslahti to describe cells that undergo apoptosis after being displaced from their anchor of ECM proteins [107]. Bruch's membrane has specific ECM proteins that are required for RPE adhesion, survival and function [34]. It is also crucial that RPE cells form tight junctions to fulfill their necessary barrier functions.

The above considerations suggest a second strategy for RPE transplantation: grow RPE on a scaffold substrate that mimics the support of the Bruch's membrane and allows the RPE to be transplanted as a fully differentiated, polarized monolayer. The idea behind the scaffold technique is that RPE cells will survive longer and remain located over the diseased retinal area, leading to greater functionality and improved visual performance. In addition, RPE grown on substrates can polarize and be implanted in the correct orientation with their apical microvilli facing the photoreceptor outer segments [108]. In AMD, Bruch's membrane deteriorates along with the RPE; therefore, transplanting healthy RPE on a biomimetic scaffold could improve the functionality and long-term survival of the RPE transplant [109].

A variety of scaffolds have been investigated. Natural biomaterial scaffolds include: human amniotic membranes [110–114], human lens capsule [115, 116], and explants of Bruch's membrane [117, 118]. Using these unaltered natural tissue supports could lead to issues with disease transmission from host and would be difficult to conform to FDA guidelines for transplantation production [119]. Natural polymers could be more advantageous and are currently being purified and used to grow hESC-RPE and iPSC-RPE. ECM proteins secreted from RPE [120] and corneal endothelial cells [120, 121], isolated gelatins and collagens [117, 120, 122–125], alginates [108], hyaluronic acid [108], fibrinogens [126], vitronectin [117, 118], laminins [117, 118, 121], and fibronectins [118, 121] all can support RPE growth. In addition to human purified proteins, a combination of ECM proteins derived from the mouse Englebreth-Holm-Swarm tumor called Matrigel is also efficient for culturing RPE [124]. The caveats to using ECM proteins are the lot-to-lot variation in composition and the difficulties to regulate production using GMP due to the animal source of the substrate.

The search for a synthetic polymer to support RPE growth and maturation has yielded several promising candidates. These compounds can be manufactured with knowledge of specific composition, are tailored to optimize RPE growth, and can be mass produced. In addition, adhesive peptides and growth factors can be embedded within the polymers to promote attachment and survival. Materials are nonimmunogenic and can be biodegradable or biostable supports. Parylene, poly(lactic-co-glycolic acid) (PLGA), poly(L-lactic acid) (PLLA), polycaprolactone, poly(glycerol sebacate), and polyhydroxyalkanoates are among those that have been investigated [108]. The polymers that successfully allowed culturing of RPE include: PLGA [127–131], PLLA [127, 131, 132], a polyethylene glycol-PLLA [129], polydimethylsiloxane [133], poly(hydroxybutyrate-cohydroxyvalerate) [134], polyether urethanes [135, 136], and parylene [137]. Parylene is already approved for use in the eye, and it can be micromachined to include ultrathin areas that mimic the permeability of Bruch's membrane. Hydrogels composed of methacrylate and (meth) acrylamide also promote RPE growth [114]. Current research is making progress toward completely xeno-free derivation of RPE from hESC and iPSC, and discovering the optimal synthetic scaffold would allow the entire process from differentiation to transplantation to be xeno-free. This approach provides benefits when creating GMP

Casaroli-Marano RP, Zarbin MA (eds): Cell-Based Therapy for Retinal Degenerative Disease.
Dev Ophthalmol. Basel, Karger, 2014, vol 53, pp 81–96 (DOI: 10.1159/000357361)

protocols and potentially eliminates complications from using animal products within the transplant [85, 138].

Clinical Trial Progression

Many groups are well on their way to bringing pluripotent stem cell-derived RPE cells to the clinic for treating ocular diseases like AMD. The first clinical trial was granted approval after long-term safety experiments and proof of concept were documented in rodents. Clinical trials began with 2 patients, one with Stargardt macular dystrophy and one with AMD. A bolus injection of dissociated hESC-RPE cells was administered into one eye of human patients. A preliminary report was published 4 months after transplantation [139]. Improved visual performance in the Stargardt disease patient was documented in the surgical eye only, but there is some controversy regarding this result [140]. The AMD patient showed improvement in the uninjected eye as well as in the injected eye, however the immunosuppressive regimen was not followed. Perhaps the most important result from this preliminary study is that patients presented no loss of vision or formation of ocular tumors. The next round of patients enrolled in the study will receive a greater number of hESC-RPE cells with the intent of increasing integration and efficiency [139]. Trials for wet and dry AMD using monolayers of hESC-RPE on scaffolds are soon to begin in London and California, and a trial using autologous iPSC-RPE has been approved in Japan. Other trials for AMD using non-RPE cells (neural stem cells and cord blood) have also been initiated. Because the eye has many advantages for developing cellular therapies, a number of groups have seized upon the opportunity. It seems likely, based on proof of concept studies in both rodent and human, that these trials have tremendous potential for the treatment of AMD as well as other ocular diseases.

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Casaroli-Marano RP, Zarbin MA (eds): Cell-Based Therapy for Retinal Degenerative Disease.
Dev Ophthalmol. Basel, Karger, 2014, vol 53, pp 81–96 (DOI: 10.1159/000357361)

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