

# Retinal Cell Transplantation, Biomaterials, and In Vitro Models for Developing Next-generation Therapies of Age-related Macular Degeneration

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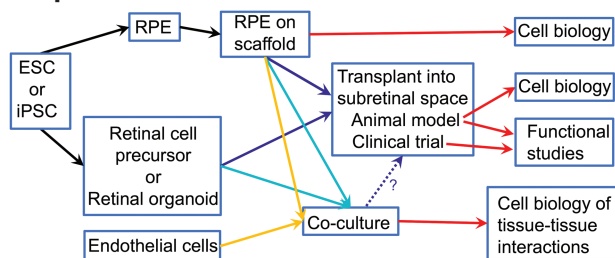
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## Abstract

Retinal pigment epithelium (RPE) cells grown on a scaffold, an RPE patch, have potential to ameliorate visual impairment in a limited number of retinal degenerative conditions. This tissue-replacement therapy is suited for age-related macular degeneration (AMD), and related diseases. RPE cells must be transplanted before the disease reaches a point of no return, represented by the loss of photoreceptors. Photoreceptors are specialized, terminally differentiated neurosensory cells that must interact with RPE's apical processes to be functional. Human photoreceptors are not known to regenerate. On the RPE's basal side, the RPE transplant must induce the reformation of the choriocapillaris, thereby re-establishing the outer blood-retinal barrier. Because the scaffold is positioned between the RPE and choriocapillaris, it should ideally degrade and be replaced by the natural extracellular matrix that separates these tissues. Besides biodegradable, the scaffolds need to be nontoxic, thin enough to not affect the focal length of the eye, strong enough to survive the transplant procedure, yet flexible enough to conform to the curvature of the retina. The challenge is patients with progressing AMD treasure their remaining vision and fear that a risky surgical procedure will further degrade their vision. Accordingly, clinical trials only treat eyes with severe impairment that have few photoreceptors to interact with the transplanted patch. Although safety has been demonstrated, the cell-replacement mechanism and efficacy remain difficult to validate. This review covers the structure of the retina, the pathology of AMD, the limitations of cell therapy approaches, and the recent progress in developing retinal therapies using biomaterials.

**Key words:** retina; retinal pigment epithelium; age-related macular degeneration; tissue scaffolds; cell transplantation; stem cells; biomaterials.

## Graphic Abstract



Retinal pigment epithelium (RPE), choroidal endothelium, and retinal precursor cells can be differentiated on scaffolds for study, and potentially, to treat retinal degenerative diseases. Co-cultures can be used to study tissue-tissue interactions. Clinical trials demonstrate safety, but modest (at best) improvement of vision. More pre-clinical studies are needed to delineate the biological mechanisms of graft/host cell interactions following transplantation.

## Introduction

Age-related macular degeneration (AMD) affects more than 11 million people in the United States and is projected to affect 22 million by 2050.<sup>1</sup> AMD is the major cause of visual impairment among the elderly. In its early phase, dry (non-exudative) AMD affects the retinal pigment epithelium (RPE) and secondarily destroys photoreceptors.<sup>2</sup> Wet (neovascular) AMD affects the RPE but often involves degeneration and aberrant growth

of the choriocapillaris and deep capillary plexus.<sup>3,4</sup> Geographic atrophy (GA) is the end-stage of the dry and sometimes wet forms and is characterized as the complete loss of photoreceptors, RPE, and macular choriocapillaris. Although a therapy exists for early wet AMD, therapies for dry AMD and GA are inadequate. Cell and tissue replacement is an emerging therapy for several retinal degenerative diseases including, and especially, dry AMD. Phase I clinical trials, which assess safety,

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demonstrate that tissue replacement therapy is safe and modest improvements in vision have been reported.<sup>5-8</sup> These successes support a move to phase II trials that test efficacy. Nonetheless, there remains a need for in-depth, long-term studies in vitro and especially in animal models to critically evaluate the biological mechanisms involved in graft-host retina interactions. The lingering and critical question remains whether the reported changes in vision are caused by cell replacement rather than other mechanisms such as neuroprotection or immunomodulation.<sup>9-12</sup> The introduction of scaffolds is a big, positive step in developing these therapies.

AMD is a multifactorial disease. Unlike gene therapy's potential to treat retinal disease caused by a single gene mutation, for example, Stargardt's disease (juvenile macular degeneration),<sup>13,14</sup> AMD is not treatable by correcting a single mutation. Scaffolds provide a platform for reconstituting RPE sheets with the neighboring choroid and neurosensory retina for preclinical studies and clinical trials. Scaffolds eliminate the potential for injected RPE cell suspensions to reflux into the vitreous cavity,<sup>8</sup> which would cause fibrosis, leading to retinal detachment and blindness. Scaffolds can organize the orientation of photoreceptor cells and ensure close juxtaposition of RPE and with photoreceptors.<sup>15,16</sup> Ideal scaffolds should provide a microenvironment that promotes (1) attachment, (2) survival, and (3) directed differentiation of RPE and/or retinal progenitor cells (RPC). For subretinal implantation, scaffolds must be nontoxic. They also need to be thin (<10  $\mu\text{m}$ ) to (1) avoid changing the focal length of the eye, (2) provide transient mechanical stability to withstand the implantation procedure, and (3) flexible to accommodate the curvature of the retina. Biodegradable scaffolds are expected to outperform the nonbiodegradable ones, as they would leave no foreign material in the subretinal space, have no impact on focal length, enable better vascularization, and enable the new RPE sheet to seamlessly merge with the host's remaining RPE. Most of the natural and synthetic scaffolds for retinal culture have been reviewed earlier.<sup>17-22</sup> Scaffolds bearing stem cell-derived cells are now in clinical trials to treat these retinal diseases. The trials have demonstrated safety and modest improvements in vision, but the trials have been too small to judge their efficacy (Table 1). They provide no data regarding whether the implants act via cell replacement, neuroprotection, immunomodulation, or some other type of mechanism.

Retinal cell and tissue replacement therapies face daunting challenges. The complex neural circuitry of the neurosensory retina must be rebuilt. Interactions of the apical RPE with photoreceptors, and basal RPE with the Bruch's membrane/choroid, must be restored, and the outer blood-retinal barrier must be re-established. We will review studies of the past several years that reveal the depth of these challenges and the substantial progress that has been made with improved scaffolds/biomaterials. We discuss recent work with scaffolds in preclinical studies and in the few clinical trials that reported outcomes for safety and visual acuity.

### Structure of the Mammalian Retina

The subretinal space separates the multilayered neurosensory retina from the RPE monolayer (Fig. 1). RPE pumps water from the subretinal space into the choroid to maintain a potential space in which the photoreceptor outer segments interdigitate with microvilli of the RPE. If fluid should accumulate, the space enlarges into a real space, forming a serous retinal detachment. The macula is ~4 mm in diameter, enriched in

cone photoreceptors for color vision, and responsible for the high-resolution vision needed for reading and for recognizing faces. Principal functions of the nonmacular retina include distinguishing light and shadow, detecting motion, and detecting low intensity light. Injecting fluid into the subretinal space creates a localized serous detachment where neo-tissue can be inserted before RPE pumps out the fluid to reattach the retina (Fig. 1A).<sup>29,30</sup>

The neurosensory retina's complex network begins processing visual inputs (Fig. 1B). Light captured by photoreceptors transmit a signal to bipolar cells and on to retinal ganglion cells whose axons form the optic nerve. Along the way, the visual inputs are refined by horizontal cells, which modulate the flow of information from photoreceptors to bipolar cells, and amacrine cells, which modulate the flow of information from bipolar cells to retinal ganglion cells. The details of how this integrated network processes visual inputs are only partially understood.

The cell body of photoreceptors is connected by a long stalk to inner segments, which house the bulk of the cells biosynthetic and catabolic machinery. The outer segments house the machinery for capturing a photon of light and converting it into an electrical signal. The outer segment is composed of a stack of disc membranes, which are synthesized every day and added to the base of the outer segment. Disc membranes at the tip are shed daily and phagocytized by the RPE (Fig. 1C). Disruption of phagocytosis leads to an accumulation of disc membranes in the subretinal space and subsequent retinal degeneration.

The fenestrated choriocapillaris, Bruch's membrane, and RPE form the outer blood-retinal barrier (Fig. 1C).<sup>21,32,34,35</sup> The RPE distributes various membrane receptors, ion pumps, and transporters specifically to either the apical or basolateral membranes. This polarity enables RPE to transport nutrients from the choriocapillaris to the neurosensory retina and transport wastes in the opposite direction. Polarity enables the RPE to maintain an ionic composition of the subretinal space that is essential for photoreceptor function. Receptor mediated endocytosis at the basolateral membrane takes up vitamin A, which is converted to 11-cis-retinal, the cofactor for the light-sensitive opsins found in photoreceptors. Interactions across the subretinal space mediate the visual cycle, whereby 11-cis- and all-trans-retinals are shuttled back and forth between RPE and photoreceptors. Apical proteins phagocytize photoreceptor outer segments (POS) shed by the photoreceptors.<sup>36,37</sup> Studies in frogs and mice indicated RPE was necessary for POS to elongate during development.<sup>38,39</sup> RPE also maintains the structure of the choriocapillaris, and both maintain Bruch's membrane. The structure and functions of Bruch's membrane continue to be active areas of research.<sup>32,40</sup> It is where drusen, a risk factor for AMD, accumulates. Drusen is a yellow conglomerate of lipids and proteins and likely impairs the permeability of Bruch's membrane. Degeneration of either the choriocapillaris or the RPE leads to degeneration of the other and the subsequent degeneration of photoreceptors.<sup>41-46</sup>

Figure 1D illustrates processes related to AMD. As RPE function degrades, illustrated by fading RPE, layers of photoreceptors are lost. With the loss of photoreceptors and visual inputs, the neural network progressively remodels to form aberrant circuits.<sup>47,48</sup> The remaining neurons can survive for some time and the inner layers of the retina remain evident histologically or by optical coherence tomography.<sup>49</sup> Eventually, the bipolar cells and interneurons will die or

**Table 1.** Clinical trials discussed this review<sup>a</sup>.

Scaffold	Size	Characteristics	# of Patients/ disease	Visual Improvement In Letters <sup>b</sup> (# of patients)	Clinical Trial	Ref
None <sup>c</sup>	N/A	hESC-RPE: 50-150,000 cells	9/Stargardt's macular dystrophy	11-15 (6)	NCT01345006	114
None <sup>c</sup>	N/A	hESC-RPE: 50-150,000 cells	8/dry AMD	12 (3)	NCT01344993	114
PET	6 × 3 mm (18.0 mm <sup>2</sup> )	hESC-RPE (~100,000) on 10 µm-thick PET, coated with plasma-derived vitronectin. Non-biodegradable	2/ wet AMD	21, 29 (2)	NCT01691261	70
Parylene	6.25 × 3.5 mm (21.9 mm <sup>2</sup> )	hESC-RPE (~100,000) on ultrathin (0.3 µm-thick), parylene membrane on a perforated, 6 µm-thick parylene support Biodegradable	5/dry AMD	17 (1)	NCT02590692	26-76
PLGA <sup>d</sup>	4 × 2 mm (8.0 mm <sup>2</sup> )	hiPSC-RPE (~100,000) on 10 µm-thick, electrospun PLGA with 350 nm mean fiber diameter. Biodegradable	None	Trial in progress	NCT04339764	65
hiPSC- secreted basal membrane	1.3 × 3 mm (3.9 mm <sup>2</sup> )	hiPSC-RPE on PET coated with a collagen-I gel; Confluent sheets released from PET with collagenase Biodegradable	2/wet AMD	No change	UMIN000011929 (Japan)	82

<sup>a</sup>Of the many clinical trials in progress, these relate to the scaffolds discussed in this review.

<sup>b</sup>Visual acuity was measured with the Early Treatment Diabetic Retinopathy Study (ETDRS) chart. An improvement of 15 letters is considered significant.

<sup>c</sup>Cell suspension. Included as a benchmark.

<sup>d</sup>Supporting rat and pig data are discussed in the text.

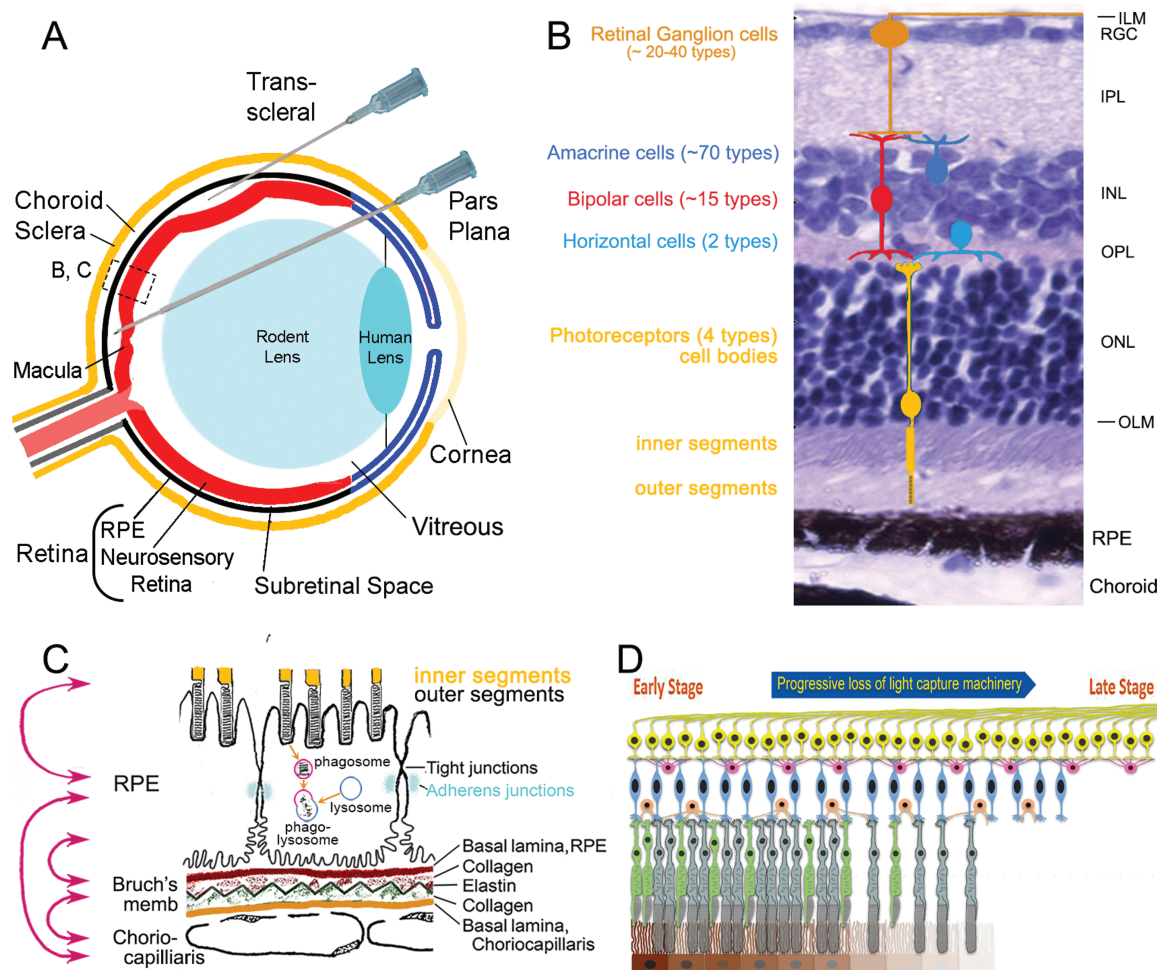
migrate away. When some photoreceptors are still present, there is a window of opportunity when transplantation of RPE, or RPE with photoreceptors, can have an effect. In that window, the neuronal networks of the INL remain intact due to continued inputs from photoreceptors. An experimental question is whether implanting new photoreceptors at a later stage would restore a disorganized neural network. Support for that hypothesis comes from a study in which cone cells, purified from hiPSC-derived retinal organoids, were implanted as a suspension into a mouse model of photoreceptor degeneration.<sup>50</sup> The implanted cells formed synapses with bipolar cells, and electrophysiologic evidence suggested the neuronal network was reorganized to some extent. AMD differs from this model because the RPE must also be replaced to restore vision. Nonetheless, this proof of concept encourages the use of biomaterials to create a layered structure of RPE and photoreceptors, as discussed in the Section “Interactions of the RPE and Neurosensory Retina.” The RGC layer will remain intact after the INL degenerates if it maintains connections with the visual cortex. An intact RGC layer provides hope for other approaches that stimulate RGC directly, such as optogenetic or retinal prosthesis technologies.<sup>51,52</sup>

### Scaffolds as a Mimetic of Bruch's Membrane: Interactions at the Basal Side of RPE

RPE can be cultured on a wide variety of scaffolds that are coated with an extracellular matrix. In vivo, RPE takes up

nutrients from the choriocapillaris through receptors and transporters located in the basolateral membranes. When grown on tissue culture plastic, nutrient media is found on the apical side of the monolayer, and RPE adapts by depolarizing the distribution of those proteins. Like most epithelia, RPE is more differentiated when cultured on a porous scaffold that is suspended in nutrient medium to expose the basolateral membranes to the medium.

To improve clinical outcomes, scaffolds were introduced to promote the survival and structural/functional integration of the transplanted RPE with the neurosensory retina and choriocapillaris. Preclinical studies examined scaffolds for their effects on the differentiation of RPE and their suitability for tissue-replacement therapy. The investigations were often limited to a cursory characterization of the RPE before advancing to clinical trials. Rigorous methods have been developed to authenticate cultured RPE.<sup>53,54</sup> These are based on morphology, electrophysiologic properties, mechanism of phagocytosis, ability to metabolize retinoids, and the expression of “signature genes,” genes whose expression is either unique to RPE or is expressed at high levels relative to other tissues.<sup>55,56</sup> Notably, proteomics confirms that expression of the corresponding proteins are comparable between cultures of highly differentiated RPE and human fetal RPE (hFRPE).<sup>57</sup> Accordingly, hFRPE from 15 to 16-week fetuses were deemed to be a gold standard. Most often, the RPE derived from hiPSC were not fully authenticated, as the analysis was limited to morphology and the expression of a few marker genes and



**Figure 1.** Structure of the retina. **(A)** The retina is a 2-layered cup formed by the RPE monolayer (black) and the neurosensory retina (red). The subretinal space is a potential space that lies between them. The choroid lies between the sclera (orange) and the RPE. The ciliary body and iris are shown in blue. The needles show 2 surgical approaches to the subretinal space. The trans-scleral approach is used in rodents due to the large lens. To insert the implant, the subretinal space is enlarged by injecting fluid to create a localized retinal detachment. RPE removes the subretinal fluid to reattach the retina. The pars plana approach to the macula is used in humans. The box is enlarged in panels (B and C). **(B)** A cartoon of the 5 major classes of neurons is superimposed on a toluidine-blue stained section of epoxy-embedded retina. **(C)** The outer blood-retinal barrier. Photoreceptor outer segments (POS), the site of phototransduction, interdigitate with apical microvilli of the RPE. The POS are composed of disk membranes that resemble a stack of coins. Daily, new discs are added to the base and old discs shed from the tip are phagocytized by the RPE. The choriocapillaris is fenestrated and separated from the RPE by the 2-4  $\mu\text{m}$ -thick Bruch's membrane. The RPE basolateral membranes are infolded. **(D)** Progression of AMD from normal (left) to GA (right). As disease progresses in the RPE, rows of photoreceptors are gradually lost. Double headed arrows, bidirectional interactions; ILM, inner limiting membrane; RGC, retinal ganglion cells; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; OLM, outer-limiting membrane. Modified with permission from Rizzolo et al. (A),<sup>31</sup> Fields et al. (C),<sup>32</sup> and Nasonkin et al. (D).<sup>33</sup>

proteins. The predominantly hexagonal morphology found in young adults in vivo is not always achieved in vitro, due to the presence of dividing cells.<sup>58</sup> Short of this, investigators should demonstrate (preferably using phalloidin with antibodies to claudin-19 and/or occludin) a polygonal lattice that has sharp borders and vertices between neighboring cells.<sup>58,59</sup> Although phagocytosis may be used as a criterion, an RPE-specific mechanism is often not documented. Occasionally, a simple measurement of transepithelial electrical resistance (TER) is made, but other electrophysiologic measures of RPE function are often ignored. These include the transepithelial potential and the apical to basal resistance ratio.<sup>19</sup> For the following studies, the depth of characterization of the RPE and in vivo results is summarized in [Supplementary Table S1](#).

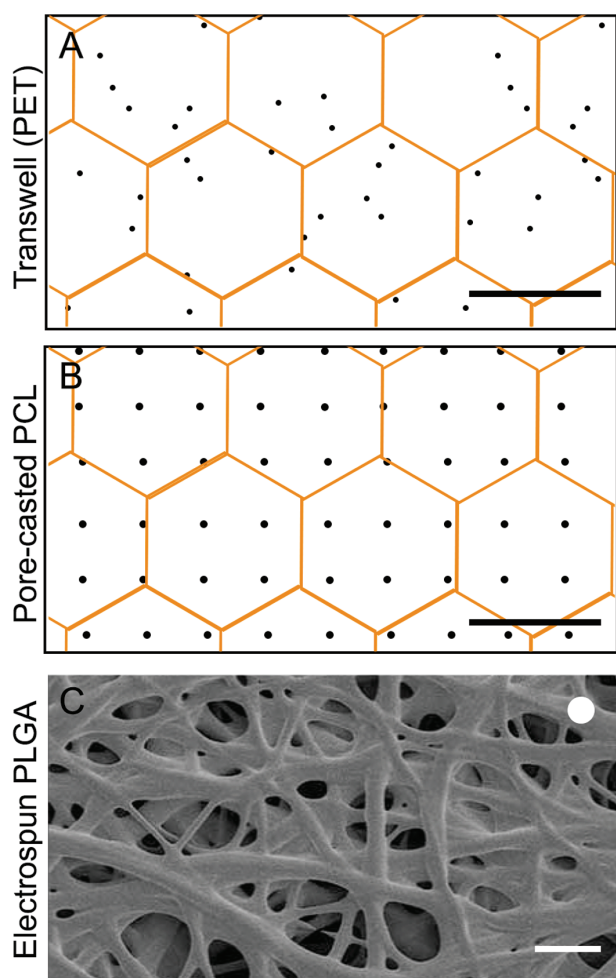
The importance of TER as a tool to evaluate RPE derived from human induced pluripotent cells (hiPSC-RPE) was

demonstrated by Ye et al.<sup>60</sup> They found that a high TER not only correlated with a mature apical junctional complex, it also correlated with the morphology of the cultures. The software to analyze morphology was developed and tested using RPE differentiated from 3 cell lines. The authors' procedure produced pure RPE that was maintained in a serum-free medium. Notably, they cultured their cells on polyethylene terephthalate (PET) coated with laminin-511 rather than the more common laminin-111 or Matrigel coating. The latter are embryonic matrixes, whereas laminin-511 is also found in mature retinal matrixes, and therefore, more appropriate for mature RPE.<sup>32</sup> A machine-learning program was trained by analyzing the subcellular localization of F-actin in cultures with high and low TER. F-actin is more reliable than ZO-1 to assess the maturity of the cytoskeleton and apical junctional complex.<sup>31,61-63</sup> The software predicted the TER of cultures

that were analyzed with phase-contrast optics, thereby providing a noninvasive way to evaluate RPE sheets prior to transplantation.

### Scaffolds Composed of Synthetic Materials

Several types of scaffolds that vary in porosity are shown in Fig. 2. In 2 examples, pores of a specified size were engineered into non-porous materials. The commonly used Transwell insert (Corning, Inc., Corning, NY) is made with a 10  $\mu\text{m}$  sheet of polyethylene terephthalate (PET). Radiation is used to etch a random distribution of pores with a diameter of 0.45  $\mu\text{m}$  pores (Fig. 2A).<sup>64</sup> Manufacturing techniques can be used to cast a uniform distribution of pores. An example is polycaprolactone (PCL) sheets cast with 0.7  $\mu\text{m}$  pores (Fig. 2B).<sup>27</sup> The grid superimposed on each image represents a monolayer of RPE to illustrate the density of pores per cell. Alternatively, electrospinning lays down a thread of defined diameter to “weave” a mesh of defined thickness and



**Figure 2.** Structure of pores on different scaffolds. (A) A schematic diagram of PET made porous by a track-etching process that left a random distribution of uniform, 0.45  $\mu\text{m}$  pores.<sup>64</sup> (B) A schematic diagram of pore-casted PCL with uniform, 0.7  $\mu\text{m}$  pores.<sup>27</sup> (C) Electrospun PLGA formed a fibrous mesh.<sup>65</sup> For comparison, the white dot indicates the size of a Transwell pore at this magnification. A lattice the size of an RPE monolayer is superimposed on panels (A and B). The image in panel (C) represents ~40% of the area occupied by a typical RPE cell. Bars A&B, 10  $\mu\text{m}$ ; Bar C, 1  $\mu\text{m}$ . Panel (C) reprinted from Sharma et al<sup>65</sup> with permission.

“pore size” as shown for poly(lactic-co-glycolic acid) (PLGA) (50:50 mixture).<sup>65</sup> This approach yields the highest density of “pores” (Fig. 2C).

The porosity/permeability of the scaffold may influence differentiation of the culture. McHugh et al<sup>27</sup> compared the PET and PCL scaffolds shown in Fig. 2. They used an hRPE preparation that was poorly differentiated on PET and found it was more differentiated on the more porous PCL scaffold. The study did not address potential effects of PCL versus PET. Nonetheless, porosity did affect RPE culture models of drusen formation. Those studies used either highly differentiated hRPE or hiPSC-RPE. One study used a scaffold of mixed cellulose ester that form a fibrous mesh akin to electrospun scaffolds. RPE secreted components of drusen that diffused into the scaffold.<sup>66</sup> Mounds of drusen were not observed. By contrast, mounds of drusen-like deposits were found when RPE was cultured on PET membranes.<sup>67-69</sup> In long-term cultures, the mounds of “drusen” increased in size and the overlying RPE monolayer was thin, as observed in AMD.<sup>68</sup> Notably, the permeability of Bruch’s membrane decreases with age, which may be a factor in AMD.<sup>24</sup> Although RPE grown on PET membranes have been implanted in animal models and humans,<sup>65,70</sup> these drusen models suggest that nondegradable, PET scaffolds may be prone to recreate the disease over time.

Permeability likely played a role when PET was compared with electrospun poly(lactic-co-glycolic acid) (PLGA) (50:50 mixture).<sup>65</sup> Using current good-manufacturing-practice protocols, hiPSC were derived from CD34+ blood cells from 3 donors with AMD and differentiated into RPE. The best PLGA scaffold was a monolayer of fused electrospun nanofibers (Fig. 2C). The resultant Young’s modulus was suitable for implantation into rat and pig models. Both cultures exhibited high fidelity to native RPE, as determined by morphology, gene expression, phagocytosis, and electrophysiologic assays. A significant difference between cultures on PET and PLGA lay in the morphology of the basolateral membranes. On PET, the basolateral membranes were flat, but on PLGA there were extensive, in vivo-like, infoldings. On PLGA, collagens IV and VIII were deposited basally, suggesting that the RPE might synthesize a de novo Bruch’s membrane as the PLGA degraded. In the best clones, VEGF was secreted principally to the basal side, which would enable the reformation of a fenestrated choriocapillaris in vivo. The lower amount of VEGF secreted apically would benefit the neural retina.<sup>71</sup>

The RPE/PLGA patch was tested in the Royal College of Surgeons (RCS) rat,<sup>65</sup> which undergoes a retinal degeneration due to a genetic defect in the RPE. The patch integrated with the host RPE. Integration was rare when a cell suspension was injected into the subretinal space. Optokinetic measurements indicated that cell suspensions and RPE patches were both beneficial, which indicates that integrated or not, neurotrophic factors appear to preserve function in the absence of an outer blood-retinal barrier. Relative to the number of cells on the patch, 10-fold more cells needed to be injected for cell suspensions to be effective.

Large-eye animal models are critical for testing the surgical approach to implant 3D patches in the subretinal space. In contrast to rodents, pigs, cats, and dogs have a macula-like region called the area centralis.<sup>72,73</sup> When tested in a pig model, substantial differences were observed between RPE patches and cell suspensions.<sup>65</sup> To selectively damage the RPE, the RPE/photoreceptor interface was disrupted with a controlled

laser burn that left the rest of the neurosensory retina intact. RPE patches on PET or PLGA preserved multiple functions, whereas suspensions of RPE were ineffective. The multifocal electroretinogram (ERG) confirmed that the N1P1 ERG signal (amplitude from the first trough to the first peak of the photopic ERG wave form) recovered over both patches. Compared to scaffold-only implants, the ONL and INL were preserved. Further, staining for rhodopsin indicated that RPE phagocytized shed outer segment discs. Once the PLGA scaffold degraded, the implanted RPE was contiguous with the host RPE. Images of the underlying choroid were not shown to evaluate whether the complex of RPE/Bruch's membrane/fenestrated choriocapillaris was restored. This study represents one of the most thorough investigations of RPE culture and subsequent implantation.

Another laboratory prepared RPE patches on thin (0.3  $\mu\text{m}$ ) sheets of parylene C.<sup>74</sup> The permeability of sheets this thin is similar to Bruch's membrane, which allows cultured RPE to draw nutrients through its basolateral membranes. For stability, the sheets were fused to a supporting mesh and coated with Matrigel. RPE derived from human embryonic stem cells (hESC-RPE) were seeded on the scaffold and formed a monolayer with apical microvilli and an apical junctional complex. However, comparison with other scaffolds was limited, because the study did not examine gene or protein expression, electrophysiology, or morphology of the basal plasma membrane. In animal studies, the hESC-RPE were cultured on parylene C scaffolds that were coated with human vitronectin and implanted in rat and pig models.<sup>25,75</sup> Twenty-one weeks after implantation into RCS rats, both scaffold-only and RPE-culture groups preserved the ONL and improved visual acuity. However, the RPE implants exhibited better preservation of inner and outer segments and more responsive sites in the superior colliculus. Rhodopsin immunoreactivity was observed in the implanted RPE. Although ultrastructural studies of the RPE microvilli and photoreceptor outer segments were not performed, the rhodopsin immunoreactivity suggests functional interactions with the photoreceptors. One month after transplantation into normal-sighted pigs, no adverse effects were observed. Phase I clinical trials were performed in patients with advanced dry AMD.<sup>26,76</sup> Four of the 5 patients enrolled in the phase I trial successfully maintained the implant (no rejection or surgical complication), and visual acuity improved modestly for one patient (Table 1).

### Scaffolds Composed of Natural Biomaterials

Amniotic membranes have been used to culture many types of cells. A current good-manufacturing-practice protocol was developed to culture RPE derived from human embryonic stem cells (hESC-RPE).<sup>77,78</sup> Gene and protein expression, phagocytosis, and cell polarity were assessed to confirm the differentiation of the cultures, but the TER was not measured. Electron microscopy revealed extensive microvilli. Despite the porous nature of the scaffold, the basal plasma membranes were flat. The principal advances were to encase the culture in gelatin to enable handling and to develop a corresponding instrument for transplantation. The cultures were compared to RPE in suspension using adult nude and RCS rats. Optokinetic and whole-field ERG analyses were performed at 5, 9, and 12 weeks. Initially, the amniotic cultures, cell suspensions, and sham implants preserved function, but with time, only the amniotic cultures sustained beneficial effects. At 12 weeks, only the amniotic cultures integrated with

the host RPE. Further, the amniotic cultures best preserved the thickness of the ONL and structure of the photoreceptors.

Three-micron thick scaffolds have been fabricated from *Bombyx mori* silk fibroin and coated with collagen-I.<sup>79</sup> Cultures on this scaffold were compared with laminin-111-coated PET. Similar results were obtained with RPE derived from 5 hiPSC cell lines. Differentiation of the RPE was confirmed by gene and protein expression. Subtle differences were observed. The cultures on fibroin synthesized more of the basal lamina components, collagen IV and TIMP3. They also appeared to engulf more POS and degrade it faster. TER and the morphology of the basal plasma membrane were not examined, and the cultures were not transplanted into animals.

A protocol was devised to let RPE synthesize its own scaffold by seeding hiPSC or monkey iPSC on to collagen-I-coated Transwell filters.<sup>80,81</sup> Four weeks post-confluence, the TER for RPE derived from 7 hiPSC lines averaged 280  $\Omega \times \text{cm}^2$ . Proteomics and gene expression for 87 signature genes was comparable to hRPE. Electron microscopy revealed apical microvilli and tight junctions, but the morphology of the basal plasma membrane was not reported (Fig. 1C). Nonetheless, polyclonal antibodies revealed that collagen IV and laminin were deposited in a neo-basal lamina. Collagenase simultaneously removed collagen-I and released a sheet of RPE from the filter. The RPE with its neo-basal lamina was implanted in RCS rats and monkeys. Safety was demonstrated, but data for retina/RPE interactions was minimal. Automation of the methodology produced cultures of similar quality.<sup>28</sup> Transplantation of these patches was reported in 2 patients with wet AMD (Table 1).<sup>82</sup> After 1 year, the visual acuity was unchanged, and the transplanted sheet remained intact with no serious safety issues.

### Summary

Two standards have been used to evaluate the effectiveness of scaffolds for the maturation of RPE. In culture studies, maturation was evaluated against the maturation of RPE on PET (which bears great similarity to hRPE).<sup>53,54,57</sup> In transplantation studies, comparisons were made against RPE on PET and RPE cell suspensions. However, culture protocols vary among laboratories, as does the depth of the analysis (Supplementary Table S1). Selecting the best scaffold would be aided by standardizing protocols of culture and analysis, as has been done for comparing differentiation protocols for retinal organoids.<sup>83</sup> Given this shortcoming, we lean toward biodegradable, porous electrospun meshes for maintaining maturation and polarization of the RPE cells. Support for this suggestion comes from the study described above that employed electrospun PLGA for culturing RPE.<sup>65</sup> These cultures exhibited the best morphology, physiology, and gene/protein expression. In vivo, the scaffold degraded, the RPE integrated with the host RPE and appeared to be functional. Nonetheless, we cannot say unequivocally that this is the best scaffold until similarly rigorous studies are made of the other scaffolds.

### Co-cultures to Study Interactions of the RPE with Neighboring Tissues

#### Interactions of the RPE and Choriocapillaris

Reliable experimental models for this interface have been slow to develop, but recent studies have made substantial progress. hRPE was cultured on the PET membrane of a

Transwell insert and suspended in a culture dish containing microvascular endothelial cells.<sup>84</sup> Thick fibrils reminiscent of the collagen layers of Bruch's membrane formed below the RPE basal lamina (Fig. 1C). Infoldings developed in the RPE basal plasma membrane. The TER increased via mechanisms involving lysyl oxidase (secreted by the endothelia) and  $\beta$ 1-integrin on the RPE. Lysyl oxidase helps crosslink collagen fibers.  $\beta$ 1-integrin transmitted signals from the extracellular matrix to increase the expression of occludin in the tight junctions. These findings were corroborated in a mouse model.<sup>84</sup>

In a second approach, RPE and a choroidal-like vasculature was cultured on a microchip.<sup>85</sup> Microchannel-patterned polydimethylsiloxane was used to culture human endothelial cells and supporting fibroblasts. Perfusible capillaries formed. ARPE-19, which form a rudimentary barrier,<sup>61</sup> were cultured on the opposite side. Barrier function increased, as measured by the transepithelial diffusion of tracers and the polarized secretion of VEGF and PEDF. Normally, most VEGF is secreted basally, but the choroidal neovascularization of wet AMD is treated by injecting anti-VEGF agents into the vitreous. In the microchip model, a high concentration of VEGF added to the apical (closest to the vitreous) side of the RPE resulted in migration of endothelial cells across the ARPE-19 monolayer, as would occur during choroidal neovascularization.

A more direct interaction was established between RPE and endothelial cells by using a hydrogel as a scaffold.<sup>86</sup> Human iPSC were used to derive RPE, endothelial cells, and mesenchymal stem cells (MSC). Variations of the following basic culture system were examined. MSC were cultured on a Transwell (PET) filter. Endothelial cells were encapsulated in a polyethylene glycol hydrogel and layered on to the MSC. RPE were cultured on top of the hydrogel layer. The RPE secreted components of Bruch's membrane basally, but the authors did not report whether infolding of the basolateral membranes had formed. Capillary-like structures were observed that required factors secreted by both the RPE and MSC. Whether these structures were capillaries or parallel cords of endothelial cells is debatable. The fenestrae-associated protein, plasmalemma-vesicle-associated protein, was evident by immunocytochemistry hinting at the presence of formed capillaries. Although the plasma membrane that faced the RPE exhibited thinned foci, these foci did not fuse with the putative luminal-facing plasma membrane to form fenestrae. Atrophy of the capillary-like structures and invasion of the RPE was induced with (1) autologous serum from AMD patients, or (2) RPE that expressed TIMP3 (Tissue Inhibitor of Metalloproteinases-3) with a mutation that is associated with AMD-like pathology.

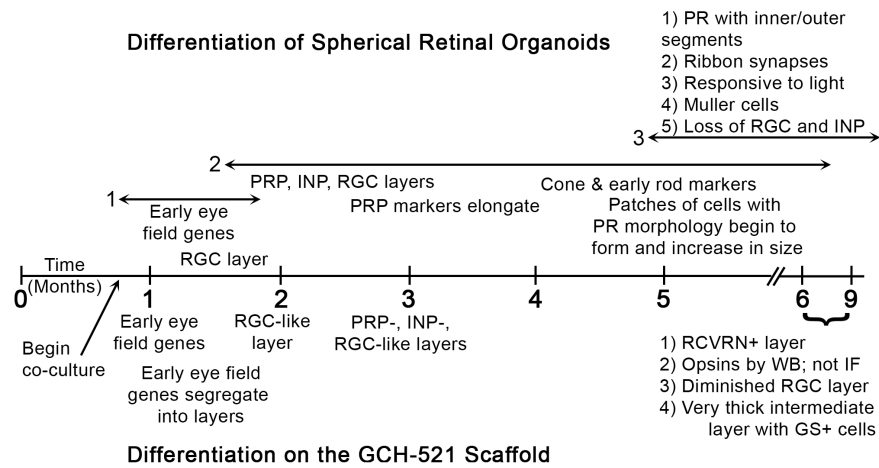
A second example of direct RPE and endothelial cells was recently reported as an unreviewed preprint on *Research Square*.<sup>87</sup> These authors extended their studies with the electrospun PLGA scaffold (see the Section "Scaffolds Composed of Synthetic Materials", Fig. 2B),<sup>65</sup> by bioprinting endothelia, fibroblasts, and pericytes on the side of the scaffold opposite the RPE. The bioprinted cells formed lumens that were perfused when the culture was implanted in rats. Electron microscopy demonstrated that co-cultures with RPE resulted in the formation of fenestra in the capillaries. RNA-sequencing demonstrated that the neo-choroid expressed RNAs for choroidal signature genes and the RPE increased expression of 46 signature genes. As the PLGA degraded, it was replaced with a thick matrix that contained many components of Bruch's membrane.

## Interactions of the RPE and Neurosensory Retina

Until recently, attempts to use scaffolds to differentiate planar, laminar retinal organoids have not been successful (eg, Yao et al<sup>88</sup>). Instead, protocols to differentiate spherical retinal organoids that manifest the layers of the neurosensory retina were inspired by the seminal work of the Sasai laboratory.<sup>89-93</sup> Recently, Capowski et al<sup>83</sup> and Singh et al<sup>94</sup> used hiPSC and hESC lines, respectively to explore the capabilities and limitations of this technology. These studies showed that mature RPE is not required to elongate photoreceptor inner segments and form rudimentary outer segment disks.<sup>39,83,91,94</sup> A limiting factor was the RPE-photoreceptor interface was not reconstituted. Consequently, growth and maturation of POS might have stalled without the structural support and niche provided by the RPE. This niche nurtures the elongation, disk shedding, and regrowth of POS and complete the visual cycle.<sup>38,39</sup> A reconstituted subretinal space would concentrate the neurotrophic factors secreted by RPE,<sup>95</sup> especially Pigment Epithelium-Derived Factor (PEDF).<sup>96</sup> PEDF is instrumental in the maturation and survival of photoreceptors.<sup>97-99</sup> The positive impact of RPE on photoreceptor maturation is suggested by the effects of the subretinal space on retinal precursors transplanted into the subretinal versus epiretinal space.<sup>100</sup> The subretinal space promoted the formation of photoreceptors, whereas the epiretinal space promoted integration with the INL and RGC layers.<sup>100</sup> Below, we outlined the recent progress in rebuilding the photoreceptor-RPE interface using in vitro, ex vivo, and in vivo studies.

The initial, straightforward approach was to co-culture retinal organoids derived from hiPSC with primary cultures of mouse RPE. The rationale was that normally, elongation of POS requires a relatively short time, and when a serous retinal detachment is resolved, POS usually reattach to RPE (Fig. 1A). Organoids isolated at different stages of differentiation (Fig. 3) were seeded on the RPE and cultured for 2 weeks. Examination of mRNA markers for the neurosensory retina revealed that only the expression of photoreceptor opsins was affected. The effect required direct contact of the organoid with the RPE. Shortcomings of the study were that the differentiation of the RPE was not assessed, and the cells were cultured on laminin-coated plastic, unable to draw nutrients from the basolateral side.<sup>101</sup>

These shortcomings were overcome by culturing RPE and mature retinal organoids on one side of a permeable microchip that was designed to have a vascular-like flow on the opposite side.<sup>103</sup> By controlling the rate of perfusion, nutrients were provided to the RPE and the wastes removed. The laminin coating of the chip facilitated the culture of hiPSC-RPE in a serum-free medium. A thin hyaluronic acid-based hydrogel separated the RPE from the retinal organoids to emulate the subretinal space. The differentiation of RPE on the microchip was verified by morphology, pigmentation, polarized secretion of VEGF, and polarized distribution of tight junction and apical membrane markers. Apical microvilli were evident but not infolding of the basolateral membrane. Nonetheless, expression of RPE markers were either comparable or enhanced on the chips. Similarly, expression of markers for the inner retinal and RGC layers were unaffected in the retinal organoids. By contrast, the expression of photoreceptor markers increased. Immunofluorescence and microscopy revealed evidence of elongated POS. The hydrogel prevented the interdigitation of POS with RPE microvilli, which might adversely affect various functions that were not explored,



**Figure 3.** Comparison of the differentiation of RPC in spherical and planar co-cultures. The timeline is in months. Above the timeline, Capowski et al<sup>83</sup> used the protocols from multiple studies to compare the time course for the differentiation of spherical retinal organoids. They observed 3 distinct stages of development, as indicated by the double-headed arrows. All times are rough approximations, as they varied with cell line and culture protocol. The characteristics of stages 1 and 2 are shown below the arrows. The characteristics of stage 3 are listed above its arrow. Below the timeline are approximate times for events observed for co-cultures of RPC cultured on the GCH-521 scaffold with RPE cultured on laminin-111 coated PET.<sup>102</sup> Reprinted from Singh et al<sup>102</sup> (CC BY-NC-ND 4.0).

such as the visual cycle. Nonetheless, there was evidence that RPE phagocytized photoreceptor disc membranes.

Because the macula is relatively flat, it would be valuable to develop a retinal organoid that was a sheet rather than a sphere. Partial success was achieved by basing the design of the scaffold on the extracellular matrix of the neurosensory retina. On day 21 of differentiation (the beginning of stage 1, Fig. 3), RPC were harvested, dissociated, and cultured on a scaffold composed of gelatin, hyaluronic acid, chondroitin sulfate, and laminin-521 (GCH-521).<sup>102,104</sup> For co-culture, RPE monolayers (hRPE or hiPSC-RPE) were established on laminin-111-coated PET. Differentiation of the co-culture is compared to the differentiation of spherical retinal organoids in Fig. 3. The differentiation protocol was like that of the spherical retinal organoids, with the exception that a cocktail of factors that included serum, retinoic acid, taurine was not added at the beginning of stage 2 (Fig. 3 top). The hypothesis was that anatomically placed RPE would replace the need for those factors. Differentiation proceeded in similar fashion to the spherical retinoids through stage 1, but thereafter the RGC layer diminished, and the intervening layer was dominated by glial-like cells (Fig. 3 bottom). Although morphologically identifiable photoreceptors were not evident, the expression of opsins increased, consistent with the co-culture studies described above. Unfortunately, the RPE and partially developed neurosensory retina simply lay side-by-side rather than interdigitating their apical surfaces. In contrast, there were notable effects on the RPE. The expression of RPE markers increased, as did the TER. The data were consistent with earlier studies of chick embryos in which the immature neurosensory retina affected the differentiation of RPE.<sup>105</sup>

To advance this model, the cocktail of factors used in stage 2 (Fig. 3, top) might be added to the basolateral chamber.<sup>83</sup> This would allow the RPE to perform its barrier function as in vivo. A further improvement would be to apply advanced methods for generating hiPSC-retinal organoids.<sup>94</sup> The goal is for co-maturation to result in elongated POS interdigitating with RPE microvilli. For more discussion of photoreceptor-RPE co-growth as a sheet, see Radisic.<sup>106</sup>

A related approach was to culture photoreceptor precursors on a planar scaffold (eg, Yao et al<sup>88</sup>). These efforts have met limited success. A recent study described a flexible, ultrathin scaffold that shows great promise.<sup>16</sup> Biodegradable poly(glycerol-sebacate) was engineered to form a thin sheet with an array of pores to orient inner and outer segments. A chamber was added above the array for seeding photoreceptor precursors isolated from stage-2 retinal organoids (Fig. 3, top). As differentiation proceeded, a multilayer of photoreceptor nuclei formed in this chamber with inner and outer segments projecting into the pores. The scaffold has the flexibility to accommodate the curvature of the retina, yet the stiffness needed for transplantation. Although no studies have been reported, the culture would be suitable for co-culture with RPE. Implanting a bioengineered, layered outer retina into the subretinal space of patients with AMD may improve upon the outcomes reported for implanting suspensions of photoreceptors in mouse models.<sup>50</sup> As a cautionary note, sheets of poly(glycerol-sebacate) that were implanted in the subretinal space of rabbits caused a selective loss of photoreceptors.<sup>107</sup>

Assuming a nontoxic substitute for poly(glycerol-sebacate) could be found, a further concern stems from an observation made in rodents. Instead of integration with the host photoreceptors, there was cytoplasmic and nuclear exchange between the implanted retinal precursors and host photoreceptors.<sup>104,108</sup> While studying this phenomenon is of interest to advance cell replacement therapies, the effect may only be transient. Studies in non-human primates and a recent study in mice indicated that integration of retinal precursors with host photoreceptors predominated.<sup>50,109</sup> Cytoplasmic/nuclear transfer may be less relevant to transplantation in patients with advanced retinal degeneration.

An alternative to RPE-photoreceptor co-culture was co-injection of RPE and photoreceptor precursors in a temperature-sensitive hydrogel composed of hyaluronan and methylcellulose.<sup>110</sup> In a mouse model, sodium iodate was used to remove the RPE and all but 4 layers of the ONL. The mixture of hydrogel and cells thinned as it was injected through a 34G needle, and re-gelled in the subretinal space.



This property would prevent cells from leaking through the retinotomy of a pars plana approach. Compared to sham and single-cell implants, co-implantation improved vision as measured by optokinetics, behavioral studies, and whole-field ERG, suggesting a potential cell replacement-based mechanism. However, neuroprotection and/or immunomodulation-based mechanisms cannot be excluded because evidence was not presented for the formation of photoreceptor and RPE layers, phagocytosis of POS, or restoration of the visual cycle.

## Summary

Inadequate attention has been paid to developing co-cultures that recreate RPE-choroid interactions and re-establishing the outer blood-retinal barrier. Recent breakthroughs have overcome the frustrations of past attempts and have already identified potential therapeutic targets.<sup>84,86,87,102</sup> These novel data support continued exploration of RPE-choroid co-cultures by *in vivo* testing to determine their potential as a tissue-replacement strategy. Further development of these models should also provide a platform to test potential therapeutic agents before committing to expensive animal studies.

The challenge facing studies of the RPE-photoreceptor interface is the difficulty of demonstrating a functional, meaningful interaction in a long-term culture and/or following transplantation.<sup>23,29,111</sup> Although POS can appear and begin to elongate without RPE,<sup>33,83,91</sup> co-culture models indicate that RPE has beneficial effects on the maturation of photoreceptors.<sup>101,103</sup> The role RPE might play in fostering maturation of photoreceptors needs further study to develop adjuvant therapies that might augment transplantation of photoreceptor precursors. In contrast to the studies of Singh et al<sup>102</sup> that used RPC (Fig. 3), Lee et al<sup>16</sup> devised a method to culture purified photoreceptor precursors on a micro-engineered scaffold. The scaffold promoted the maturation of polarized photoreceptors as a flat sheet. This approach may be more suitable for grafting and for RPE-photoreceptor co-culture. Hopefully, co-culture data will soon emerge from this group.

## Future Directions: The Conundrums Facing Retinal Tissue Replacement Therapy

Phase I clinical trials of RPE on scaffolds demonstrated safety with modest improvements of visual acuity and encourage larger phase II trials to test efficacy. Nonetheless, successes and failures are difficult to interpret in clinical trials, where tissue cannot be examined directly by routine high-resolution histological and immuno-histological techniques. There is insufficient information from *in vitro* and laboratory animal studies where detailed analyses can be performed postmortem. Several of the studies listed in [Supplementary Table S1](#) exhaustively evaluated the ability of their scaffold to generate highly differentiated RPE. These studies are valuable to help narrow down which scaffolds would best promote the maturation of implanted RPE and to delineate biological mechanisms that impact the results obtained *in vivo*. Left unanswered is whether it is better to transplant immature or mature RPE to rebuild the RPE-photoreceptor outer segment niche. One might predict that mature RPE have higher potential for achieving positive clinical results, especially when co-grafted with RPC, because in humans, RPE matures before RPC. Mature RPE may have a better potential to mature RPCs and control proliferation. However, 2 studies suggest

that it is better to transplant immature RPE,<sup>112,113</sup> as these cells are more developmentally plastic and might better survive during transplantation. Most investigations listed in [Supplementary Table S1](#) have not rigorously determined the maturity of the RPE used for transplantation in laboratory animals or in patients.

Although cell suspensions were not as effective as planar sheets of RPE in laboratory animals, the issue needs to be revisited. Cell suspensions modestly improved vision in a clinical trial that focused on safety (Table 1).<sup>114,115</sup> This approach has the advantage that a much smaller cannula size lessens the negative surgical impact on the neurosensory retina. However, when the cannula is withdrawn, reflux of transplanted cells into the vitreous cavity is common because the retinotomy size is large enough for cells to spill into the vitreous cavity (Fig. 1A).<sup>8</sup> Those cells can form a fibrotic scar on the inner (vitreous) surface of the retina. The scar can exert traction to trigger proliferative vitreoretinopathy and cause a retinal detachment.<sup>116,117</sup> To avoid this complication, a procedure was developed to use the trans-scleral approach to enter the subretinal space. Under optical guidance, the catheter is threaded through the subretinal space until the macula is reached.<sup>118,119</sup> Although this approach was originally developed to inject umbilical cord cells, it could be used to deliver cell suspensions of RPE or RPC. To summarize thus far, head-to-head comparisons of the various scaffolds have not been done, and the efficacy of cell suspensions needs to be revisited.

A greater understanding of the biological mechanisms, driving the establishment of the RPE/photoreceptor interface is needed in both long-term *in vitro* and *in vivo* experiments. For cell-replacement mechanisms to account for clinically significant improvements in vision, photoreceptor outer segments need to interdigitate and interact with RPE microvilli on a large scale. Transplant models for RPE and clinical trials show minimal evidence for restored photoreceptor-RPE interactions regardless of whether the RPE was implanted as a sheet or a suspension. In laboratory animal and clinical studies, optical coherence tomography revealed that the implanted RPE was closely juxtaposed to the recipient's neuroretina. Occasionally small changes were reported in the ellipsoid zone of photoreceptor inner segments. The accumulated data on transplantation hint at potential roles for other mechanisms that modulate the results, such as neuroprotection.<sup>9,10</sup> Neuroprotection contributed to preservation of the ellipsoid zone in patients with macular degeneration.<sup>120</sup> Immunomodulatory mechanisms can contribute to rescuing degenerating photoreceptors.<sup>11,12</sup> Likewise, transplanted RPE might enable some host RPE cells to undergo division and contribute to the outcomes.<sup>10</sup> Furthermore, immature (partially differentiated) RPE expressed higher levels of neuroprotective factors and were superior to fully differentiated RPE in the RCS rat model.<sup>112</sup> These findings make it even harder to parse neuroprotection versus immunomodulation versus cell replacement-based mechanisms.

Clinicians are exploring the neuroprotective effect as a transient solution to attenuate the onset of blindness caused by the degeneration of photoreceptors. As factories that provide an ongoing source of neuroprotective factors, umbilical tissue-derived cells were implanted in the subretinal space, as described above.<sup>119</sup> Although vision did not improve, the trial was performed on a patient with advanced GA and only existing photoreceptors at the edges of the atrophied region

would be able to respond. Another approach avoids incising or puncturing the retina by transplanting human fetal retinal cells into the vitreous as epiretinal grafts (NCT03073733). In this phase IIB trial of 37 patients with retinitis pigmentosa (a collection of over 3000 genetic retinal degenerations), visual acuity improved by 37 letters.<sup>121</sup> An improvement >15 letters is considered clinically significant. Therefore, the mechanisms of neuroprotection likely make a major contribution to the effects of transplanted RPE patches or cell suspensions.

Co-culture models can explore some of the complex issues that surround transplantation and might also provide transplantable tissue.<sup>16,33,102,122</sup> The impetus for this approach comes from the pioneering studies by the team of Drs. Aramant and Seiler.<sup>123</sup> In animals that suffered complete loss of their photoreceptors, implanted sheets of fetal neurosensory retina plus RPE improved vision. These studies led to promising phase II trials for retinitis pigmentosa and dry AMD (NCT00345917, NCT00346060).<sup>124</sup> The hope is that rare, fetal tissue will be replaced by continued development of hiPSC-derived planar sheets of RPE with neurosensory retina.<sup>104,125</sup> With the help of flexible, biodegradable scaffolds, there is great promise for this next generation of 3D transplants.<sup>16,126</sup>

Scaffolds for retinal cell replacement is a rapidly developing, promising direction for translational ophthalmology research. Although still in the experimental stage, scaffolds have rapidly found their way into pilot clinical trials. Surgical techniques for impeccably implanting RPE and neuronal patches<sup>26,76</sup> are outpacing our understanding of retinal biology. Advances in surgical delivery solves the mechanistic (surgery) and regulatory (safety) problems of retina replacement, but not the biological (efficacy) problems, including restoring retinal circuitry, reestablishing retinal interactions across the subretinal space, and reestablishing the outer blood-retinal barrier. Improved retina replacement strategies can be developed by solving these problems with laboratory animals and in vitro models. Increasingly sophisticated culture and animal models with improved synthetic and natural scaffolds are closing the gap between surgery and biology. Scaffolds that promote and maximize the functions of retinal patches, including functional RPE-photoreceptor interactions, will continue to play a critical role in developing novel tissue-replacement therapies to ameliorate impaired vision.

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## Conflict of Interest

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## Author Contributions

L.J.R.: Conception and design, manuscript writing, final approval of manuscript. I.O. N.: Conception and design, manuscript writing. R.A.A.: Manuscript writing.

## Data Availability

No new data were generated or analyzed in support of this research.

## Supplementary Material

Supplementary material is available at *Stem Cells Translational Medicine* online.

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