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Concise Review: Update on Retinal Pigment Epithelium Transplantation for Age-Related Macular Degeneration

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Key Words. Autologous stem cell transplantation • Cell transplantation • Clinical trials • Experimental models • Induced pluripotent stem cells • Embryonic stem cells • Retina

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ABSTRACT

Retinal cell therapy can have the objectives of rescue (i.e., modulation of metabolic abnormalities primarily for sight preservation) as well as replacement (i.e., replace cells lost due to injury or disease for sight restoration as well as preservation). The first clinical trials of retinal pigment epithelium (RPE) transplantation for vision-threatening complications of age-related macular degeneration (AMD) have begun with some preliminary signs of success (e.g., improvement in vision in some patients, anatomic evidence of transplant-host integration with some evidence of host photoreceptor recovery, long-term survival of autologous induced pluripotent stem cell-derived RPE transplants without immune suppression) as well as limitations (e.g., limited RPE suspension survival in the AMD eye, limited tolerance for long-term systemic immune suppression in elderly patients, suggestion of uncontrolled cell proliferation in the vitreous cavity). RPE survival on aged and AMD Bruch's membrane can be improved with chemical treatment, which may enhance the efficacy of RPE suspension transplants in AMD patients. Retinal detachment, currently used to deliver transplanted RPE cells to the subretinal space, induces disjunction of the first synapse in the visual pathway: the photoreceptor-bipolar synapse. This synaptic change occurs even in areas of attached retina near the locus of detachment. Synaptic disjunction and photoreceptor apoptosis associated with retinal detachment can be reduced with Rho kinase inhibitors. Addition of Rho kinase inhibitors may improve retinal function and photoreceptor survival after subretinal delivery of cells either in suspension or on scaffolds. STEM CELLS TRANSLATIONAL MEDICINE *2019;8:466–477*

SIGNIFICANCE STATEMENT

Cell-based therapy can be effective in chronic degenerative retinal disease, but the following obstacles must be addressed to optimize therapeutic efficacy: the abnormal microenvironment of the host, surgery-induced changes in the host retina, and the need for immune suppressive therapy for subretinal allogeneic retinal pigment epithelium (RPE) transplants.

INTRODUCTION

Age-related macular degeneration (AMD) is the leading cause of blindness among persons older than age 55 years in the industrialized world [1]. Patients can experience profound loss of central vision, which compromises their ability to read, recognize faces, drive an automobile, and live independently. Severe central visual loss can occur as a result of pathological capillary growth from the choroidal circulation into the subretinal pigment epithelium and subretinal spaces. These vessels, termed choroidal new vessels (CNVs), bleed and fibrose, which results in photoreceptor atrophy. Because the CNVs grow primarily under the macula, which is the part of the retina that supports high acuity vision, they cause severe central vision loss. The other mechanism of central vision loss in AMD patients is geographic atrophy (GA). This process is characterized by photoreceptor apoptosis, retinal pigment epithelium (RPE) atrophy, and choriocapillaris atrophy [2]. Like CNVs, GA involves primarily the macula of AMD patients. Both CNV and GA are preceded by inflammation involving the RPE, choriocapillaris, Bruch's membrane complex [3]. Bruch's membrane is largely acellular tissue consisting mainly of collagen and elastin interposed between the RPE and choriocapillaris and on which the RPE reside in situ.

Both forms of late AMD can exist in a single eye [4]. Factors that drive progression to CNV formation versus GA are unknown, and their identification is an area of intense investigation [5–7].

Antivascular endothelial growth factor antibodies are highly effective in preventing visual loss due to CNVs [8–12]. There is no established treatment, however, for patients with GA. The landscape of options under study includes complement pathway inhibitors (which block complement activation and are intended to reduce the inflammatory response in the RPE-choriocapillaris that is believed to underlie AMD progression), visual cycle inhibitors (which block *cis*-retinal synthesis and are intended to reduce lipofuscin formation in the RPE, thus reducing oxidative damage), intravitreal neurotrophic factors (intended to reduce photoreceptor apoptosis in GA), lipid metabolism modulators (intended to modulate lipid deposition in Bruch's membrane, thus reducing progression of AMD to development of GA), and cell-based therapy (intended to replace damaged RPE and/or photoreceptors in patients with advanced CNVs or GA).

The purpose of this review is to provide an update on the use of cell-based therapy to treat vision-threatening complications of AMD. A number of cell types are under clinical study including RPE cells, neural stem cells, and bone-marrow derived stem cells (Table 1). Cell transplants can be used as "rescue therapy." Rescue involves preservation and, in some cases, restoration of function in tissue that is destined to die or malfunction due to an underlying disease. Rescue therapy may be sight-restoring to the degree that dying cells, which cannot support vision, can return to normal physiological function. Cell transplants can also be used as "replacement therapy." Replacement involves providing healthy cells (e.g., RPE) to replace those that are dead or dying with the goal of restoring physiological function to a tissue or organ. In the case of RPE transplants for GA, one attempts to replace the RPE cells lost due to GA with the goal of preventing the spread of atrophy and additional visual loss. Areas of GA may harbor some residual photoreceptor nuclei [4, 13, 14], so some recovery of visual function is also possible depending on the extent of damage prior to the transplant. As discussed elsewhere, the eye is a particularly suitable target for cell therapy in the central nervous system [15]. This review will focus on results with RPE cells, as currently the earliest phase clinical trial data are available primarily for this cell type in the treatment of AMD.

Clinical Studies

RPE Transplantation for Choroidal Neovascularization in AMD

Tezel et al. transplanted sheets of adult allogeneic RPE in conjunction with surgical removal of subfoveal CNVs in AMD patients [16]. Different cadavers were used to provide RPE for different patients. Tissue was prepared according to a previously established protocol [17], and the method has been described in detail elsewhere [18]. Briefly, before RPE harvesting, gelatin blocks were cut into triangular pieces and mounted on a vibratome. Gelatin sheets 100-μm thick were cut from the blocks and kept in CO_2-free medium at 4°C. Allogeneic adult human RPE cells were harvested as intact sheets from cadaver eyes obtained within 24 hours of death. Specifically, the sclera was peeled away from the choroid, and the globe was incubated with 25 U/ml Dispase (Gibco, Grand Island, NY) for 30 minutes. Eyes were rinsed with CO_2-free medium, and a circumferential incision was made into the subretinal space along the ora serrata. Loosened RPE sheets were removed from the rest of the ocular tissue and placed on a slice of 50% gelatin containing 300 mM sucrose with the apical surface of the RPE facing upward. The gelatin film with the adult human RPE sheet was then incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37°C for 5 minutes to allow the gelatin to melt and encase the adult RPE sheet. The specimen was kept at 4°C for 5 minutes to solidify the gelatin and stored in CO_2-free medium at 4°C for up to 24 hours before transplantation to the subretinal space. Positive identification of the cells using immunohistochemistry confirmed their epithelial origin, and viability of the RPE sheet was assessed using standard methods. All 12 patients were treated with triple immune suppression (corticosteroids, azathioprine, cyclophosphamide) preoperatively and postoperatively. One year after surgery, there was no significant change in best-corrected visual acuity, contrast sensitivity, or reading speed. Although there was no sign of transplant rejection among patients who were able to continue immunosuppressants for 6 months, six patients did not tolerate the regimen, and apparent rejection (i.e., graft fibrosis) was observed soon after the immune suppressive regimen was stopped in three patients. Relevant complications included migration of the graft to an extrafoveal location (one patient), retinal detachment with proliferative vitreoretinopathy (one patient), and epiretinal membrane formation distorting the macula and requiring additional surgery (two patients).

Binder et al. reported the results of RPE transplants for AMD patients with subfoveal CNVs in a prospective controlled trial [19]. Patients underwent CNV excision followed by subretinal injection of autologous RPE in suspension. As described previously, the RPE cells were harvested subretinally from the nasal side of the optic disc and given to a pathologist, who centrifuged the freshly harvested RPE cells, counted them in a hemocytometer, and evaluated them under a microscope [19, 20]. If there were at least 1,500 RPE cells, then they were diluted in 0.1–0.2 ml physiologic saline and returned to the surgeon for transplantation. Controls underwent subretinal CNV excision without transplantation. Reading speed was superior in the transplant group by 12 months follow-up, and there was a trend for better visual acuity using Early Treatment Diabetic Retinopathy Study (ETDRS) visual acuity charts [21] in the transplant group.

Lu et al. reported the results of autologous RPE sheet transplants in AMD patients undergoing CNV excision [22]. RPE harvesting was as follows [22]. After the submacular CNV was excised, the detached RPE layer around the CNV was exposed. If the initial area of RPE detachment was not wide enough to harvest a free graft, balanced salt solution was injected into the subRPE space using a 39-gauge needle (E7365; Bausch & Lomb, Tampa, FL) to expand the area for harvesting a graft of adequate size. The separated RPE monolayer was cut with microscissors. The free graft was gently transposed to the presumed submacular site and was fixed into position by gently pressing the edge of the graft with the smooth back surface of the microforceps as well as by using a heavier-than-water perfluorocarbon liquid. The retina flap, used to create access to the subretinal space and CNV, was then flipped back into position after the perfluorocarbon

Table 1. Human cell therapy trials for late-stage age-related macular degeneration

Disease (ClinicalTrials.gov identifier)	Phase	Cell type transplanted	Center (PI)	Sponsor
AMD-GA (NCT01344993, NCT02563782, NCT02463344)	I/II	ESC-RPE (MA09-hRPE)	Jules Stein-UCLA (Schwartz) Wills Eye Hospital (Regillo) Mass. Eye & Ear Infirmary (Eliott) Bascom Palmer Eye Institute (Rosenfeld)	Astellas Pharma
AMD-GA (NCT03178149)	Ib/II	ASP7317 (MA09-hRPE)	Jules Stein-UCLA Retina Specialty institute New Jersey Retina Mid-Atlantic Retina	Astellas Institute for Regenerative Medicine
AMD-GA (NCT01674829)	I/II	ESC-RPE (MA09-hRPE)	CHA Bundang Medical Center (Song)	CHA Bio & Diostech
AMD-GA (NCT03305029)	Interventional	SCNT-ESC-RPE	CHA Bundang Medical Center (Song)	CHA University
AMD-CNV (NCT01691261, NCT03102138)	I	PF-05206388 (ESC-RPE on a polyester membrane)	University College London (Pfizer)	Pfizer
AMD-GA or CNV (NCT02464956)	Observational	Autologous iPSC-RPE	Moorfields Eye Hospital	Moorfields Eye Hospital NHS Foundation Trust
AMD-GA (NCT02590692)	I/II	ESC-RPE on a polymeric substrate (CPCB-RPE1)	Retina Vitreous Associates Medical Group (Rahhal) USC Keck School of Medicine (Kashani) Southern California Desert Retina Consultants Orange County Retina Medical Group California Retina Consultants	Regenerative Patch Technologies, LLC
AMD-CNV	Interventional	Autologous iPSC-RPE	Riken Institute for Developmental Biology (Takahashi)	Riken Institute for Developmental Biology
AMD (NCT00874783)	Observational	iPSCs	Hadassah Medical Organization (Reubinoff)	Hadassah Medical Organization
AMD-GA (NCT02286089)	I/II	ESC-RPE	Hadassah Ein Kerem University Hospital (Jaouni) Rabin Medical Center (Erlich) Kaplan Medical Center (Morori-Katz) Tel Aviv Souraski Medical Center (Barak) Retina Vitreous Associates (Boyer) Byers Eye Institute (Do) Retina Consultants Medical Group (Telander) West Coast Retina Medical Group (McDonald)	Cell Cure Neurosciences, Ltd.
AMD-GA (NCT02749734)	I/II	ESC-RPE	Southwest Hospital (yin)	Southwest Hospital, Chongqing, China
AMD-GA (NCT03046407)	I/II	ESC-RPE	First Affiliated Hospital of Zhengzhou University (Qi)	Chinese Academy of Sciences
AMD-GA (NCT02755428)	I/II	ESC-RPE	Beijing Tongren Hospital (qi)	Chinese Academy of Sciences
AMD-GA (NCT02868424)	I	Fetal human RPE	First Affiliated Hospital with Nanjing Medical University (Liu)	First Affiliated Hospital with Nanjing Medical University, Nanjing, China
AMD-GA (NCT02016508)	I/II	Bone marrow-derived SCs	Al-Azhar University (Safwat)	Al-Azhar University
AMD (NNCT01920867)	Interventional	Bone marrow-derived SCs	Retina Associates of South Florida (Weiss)	Retina Associates of South Florida

(Continues)

Table 1 (*Continued*)

Disease (ClinicalTrials.gov identifier)	Phase	Cell type transplanted	Center (PI)	Sponsor
AMD-GA (NCT01736059)	I	Bone marrow-derived CD34+ SCs	University of California, Davis (Park)	University of California, Davis
AMD-GA or CNV (NCT01518127)	I/II	Autologous bone marrow-derived SCs	University of Sao Paulo, Brazil (Siqueira)	University of Sao Paulo
AMD-GA (NCT01632527)	I/II	HuCNS-SC	Retina Foundation of the Southwest Retina Vitreous Associates Medical Group Byers Eye Institute New York Eye and Ear Infirmary Retina Research Institute of Texas	StemCells, Inc.
AMD-GA (NCT01226628)	I	CNTO 2476 (umbilical tissue-derived cells)	Wills Eye Hospital (Ho)	Janssen Research & Development, LLC
AMD-GA (NCT00447954)	II	NT-501 implant	Retina-Vitreous Associates Medical Group Retina Group of Florida Bascom Palmer Eye Institute Ophthalmic Consultants of Boston Beaumont Eye Institute Retina Foundation of Southwest Vitreoretinal Consultants University of Utah	Neurotech Pharmaceuticals

Abbreviations: AMD, age-related macular degeneration; CNV, choroidal neovascularization; ESC-RPE, human embryonic stem cell-derived RPE; iPSC-RPE, human induced pluripotent stem cell-derived RPE; GA, geographic atrophy; RPE, retinal pigment epithelium; SCNT-ESC-RPE, somatic cell nuclear transfer human embryonic stem cell derived-RPE; NT-501 implant, transformed RPE that overexpress ciliary neurotrophic factor.

was removed. Vision improved from 16.61 $\pm$ 27.98 ETDRS letters to 29.16 $\pm$ 23.80 letters (p = .02) during a mean follow-up period of 21.72 $\pm$ 11.09 months, but the series was retrospective with the potential for confounding by a number of factors including case selection and concomitant cataract surgery. Fifteen ETDRS letter improvement corresponds to doubling of the visual angle, which is generally accepted as a clinically significant degree of change in vision [23].

Mandai et al. transplanted a sheet of autologous induced pluripotent stem cell (iPSC)-derived RPE into the subretinal space of an AMD patient who also underwent surgical excision of a subfoveal CNV [24]. iPSCs were generated from skin fibroblasts using nonintegrating episomal vectors carrying *GLIS1, L-MYC, SOX2, KLF4, OCT3/4* and differentiated into RPE as described previously [25, 26]. Pigmented colonies of RPE were picked manually and cultured to confluence. The pigmented cells were verified as RPE based on their ultrastructural appearance and based on biochemical features (e.g., presence of retinoid cycle enzymes [RPE65], cellular retinaldehyde binding protein [CRALBP], phagocytosis proteins [MERTK], chloride channels [BEST1], and tight junction proteins [ZO-1] as determined by reverse transcription polymerase chain reaction and immunohistochemistry). In addition, iPSC-derived RPE transepithelial resistance was measured as was the ability of the RPE to phagocytose porcine rod photoreceptor outer segments. The autologous iPSC-derived RPE cells were assessed for quality and safety before transplantation, and whole-genome sequencing, whole genome methylation profiling, and expression analyses were also performed. To generate RPE sheets without a scaffold, iPSC-RPE were seeded on collagen gel and cultured in RPE cell sheet medium. After reaching confluence, the iPSC-RPE was cultured in serum-free retinal medium supplemented with basic fibroblast growth factor and SB431542 (0.5 mM) for at least 4 weeks. The medium was changed every 2–3 days. To prepare iPSC-RPE cell sheets without any artificial scaffold, the insert membrane was removed and collagenase I was applied at 37°C for 30 minutes to dissolve the collagen gel. The iPSC-RPE sheet was then cut at the margin to release it from the insert as an intact cell sheet. The iPSC-RPE cell sheets were washed in phosphate-buffered saline and transferred to a dish. These sheets were kept moist with Dulbecco's modified Eagle's medium/F12 (200 ml) until they were cut using laser microdissection. The RPE sheets were prepared for transplantation on the day of surgery. The RPE sheet was cut in one corner so that the apical surface could be identified intraoperatively. The 1.3 mm $\times$ 3 mm RPE sheet was delivered to the subretinal space using a modified 20-gauge cannula. One year after surgery, the sheet seemed to be intact; however, there was no improvement in the patient's vision (stable at 20/200). Given the degree of foveal atrophy evident before surgery, this result is not surprising. There was no clinical or angiographic evidence of graft rejection in this patient, who was not immune suppressed.

da Cruz et al. reported the use of human embryonic stem cell (hESC)-derived RPE transplants to treat two AMD patients with subfoveal CNVs associated with significant subretinal hemorrhage [27]. The hESCs were expanded on vitronectin-coated culture dishes and spontaneously differentiated into pigmented RPE cells that were manually isolated and passaged. With immunohistochemistry and transmission electron microscopy, these cells exhibited typical features of mature RPE such as expression of CRALBP, BEST1, ZO-1, pigment epithelium-derived factor, premelanosomes, and apical-basal polarization. In addition, they phagocytosed photoreceptor outer segments. A 6 mm $\times$ 3 mm patch of a well differentiated RPE monolayer

resting on a vitronectin-coated polyester membrane was transplanted into the subretinal space and positioned under the macula. Patients were immune suppressed with perioperative oral prednisone and intravitreal implants providing sustained delivery of fluocinolone acetonide. One patient developed a severe retinal detachment after the transplant procedure and underwent successful retinal reattachment surgery. In the patient with the least foveal atrophy before surgery, vision improved 29 letters on the ETDRS vision chart, from 20/640 to 20/160 (normal = 20/20), and reading speed improved from 0 words per minute to ~80 words per minute (normal = 200 words per minute) by 12 months after surgery. In the patient with the postoperative retinal detachment, who had more profound foveal atrophy before the transplant procedure, vision improved 21 ETDRS letters, from 20/800 to 20/150, and reading speed improved from 0 words per minute to ~50 words per minute by 12 months after surgery. Because vision can improve after subretinal surgery alone in this setting, with approximately 25% of eyes improving 10 or more ETDRS letters, and because there were no control surgeries in this series, one cannot ascribe these improvements to the transplants with complete certainty [19, 22, 28, 29]. There was, however, anatomic evidence of integration of the RPE transplant with host retina and focal improvement in photoreceptor anatomy over the transplant in both patients.

RPE Transplantation for GA in AMD

Schwartz et al. reported the use of ESC-derived RPE suspensions to treat nine AMD patients with GA [30–32]. A hESC line (MA09) was used to generate a master cell bank that was thawed and expanded on mitomycin-C-treated mouse embryonic fibroblasts for three passages. After embryoid body formation and cellular outgrowth, pigmented RPE patches were isolated with collagenase. The RPE cells were isolated, purified, expanded, and cryopreserved at passage-2 for clinical use. RPE suspensions were delivered to the subretinal space via a small retinotomy (38-gauge) and were directed to the border area of GA. Patients were immunosuppressed with low dose tacrolimus and mycophenolyate mofetil before surgery and for 6 weeks after surgery. At week-6, tacrolimus was to be discontinued with continuation of mycophenolate for an additional 6 weeks. Complications were associated with this regimen in the AMD patients indicating that older patients probably will not tolerate sustained systemic immune suppression [33]. After surgery, the transplants seemed to expand with increased pigmentation in the subretinal space. There was no evidence, however, of significant expansion of pigmented tissue into the area of GA. Pigmented cells seemed to expand away from and surrounding the area of GA but not into it. Two of nine patients developed pigmented epiretinal membranes (i.e., growth of pigmented cells in a sheet on the surface of the retina) that were not clinically significant. Among the eight eyes that did not develop cataract after surgery, median improvement in best-corrected visual acuity was 14 letters by month 12 (interquartile range [IQR] 3.0–19.0 letters). Fellow eyes lost a median of one letter by month 12 (IQR −5.0 to +6.1 letters). Potential confounders in the interpretation of these results include: lack a of control group receiving subretinal fluid injection without cells (sham surgery can have a rescue effect), bias (neither the examiner nor the patient were masked regarding the treatment received) [34], and improvement due to repeat testing [35]. There was no correlation between the presence of postoperative pigmentation and postoperative visual improvement, nor did the absence of hyperpigmentation preclude visual improvement.

Kashani et al. transplanted 3.5 mm × 6.25 mm sheets of hESC-derived RPE monolayers into areas of GA in five AMD patients [36]. Since RPE cells are lacking in areas of GA, these experiments explicitly replace lost native RPE in the area of GA. The RPE spontaneously differentiated from an ESC line (NIH-H9) through removal of soluble growth factors. Second-passage hESC-RPE was cryopreserved as an intermediate cell bank [37, 38]. The authors nanoengineered a parylene C scaffold for RPE sheet delivery [39]. The scaffolds are 6 μm thick to provide mechanical support with 0.3 μm thick, 40 μm diameter diffusion zones to facilitate nutrient transfer from the choriocapillaris. The diffusion zones occupy ~60% of the scaffold surface area. Passage-3 RPE were seeded onto one of these vitronectin-coated parylene-C membranes and grown to confluence for ~4 weeks, achieving a density of 10^5 cells per scaffold. Before transplantation, the scaffolds were checked for confluence, pigmentation, and cobblestone morphology. These cells demonstrate RPE markers (e.g., RPE65) as well as phagocytosis of photoreceptor outer segments [38]. The scaffold could not be implanted in one patient due to the presence of fibrinoid debris in the subretinal space. Surgery for the other four patients was uneventful with delivery of the RPE-scaffold into the area of GA. In these latter patients, the appearance, location, and size of the implants did not change during follow-up, which ranged from 120 to 365 days (mean 260 days) after surgery. Four of the five subjects showed no substantial change in vision from baseline. One transplant recipient, however, improved by 17 ETDRS letters, and this improvement was maintained at last follow-up (day 120 after surgery). Contralateral fellow eyes showed no significant change in vision or worsened during follow-up. This degree of visual improvement is almost never observed in GA patients [40], so it seems likely that the improvement was related to the surgical procedure. Normally, the eye fixates on an object by moving so that the image of regard falls on the fovea. When the fovea is damaged, fixation becomes unsteady and involves extrafoveal fixation points [41]. Two subjects developed stable fixation over the implant.

Optical coherence tomography (OCT) provides 3-μm resolution images of the retina and adjacent structures (i.e., RPE, Bruch's membrane, choriocapillaris) [42, 43]. Four highly reflective bands in OCT images of the outer retina have been identified as the external limiting membrane (ELM; comprising heterotypic adherens junctions between photoreceptor inner segments and the Muller cell apical processes), the ellipsoid zone (comprising the outer portion of photoreceptor inner segments enriched in mitochondria), the interdigitation zone (corresponding to the contact cylinder, a structure defined by ensheathment of the cone outer segments by the apical processes of the RPE), and the RPE band [44, 45]. Changes in outer retinal OCT images associated with different types of photoreceptor pathology are consistent with this classification [46, 47]. Recovery of visual acuity following successful macular hole surgery is associated with restoration of the ELM [48]. Visual recovery following antivascular endothelial growth factor therapy for CNVs in AMD patients is associated with restoration of the ellipsoid zone, which occurs where the ELM is retained [49]. Kashani et al. noted that OCT images demonstrated formation of an ELM over the transplant in

four patients (including the patient with marked visual improvement), which may mean that the transplanted RPE cells were integrating with the overlying host photoreceptors [14, 36, 47]. One may question whether visual improvement is possible in areas of GA, but, as noted above, histopathological studies have demonstrated that some photoreceptor cells lacking inner and outer segments persist in these areas [4, 13, 14]. Some studies indicate that relatively few photoreceptors are needed to support functional vision [50]. Thus, a rescue effect may underlie the visual improvements noted in this study.

Preclinical Studies

The results of the studies cited above are consistent with a modest treatment benefit for patients with the exudative and atrophic complications of late AMD. As these studies are early phase clinical trials, the subjects enrolled tend to have advanced disease with limited potential for visual improvement. Nonetheless, the results available indicate several potential obstacles to clinical translation on a large scale. Potential obstacles include: (a) RPE survival on Bruch's membrane in AMD eyes; (b) cell delivery to the subretinal space; (c) physiological cell behavior following transplantation (e.g., integration with host retina, prevention of retinal detachment-induced synaptic disjunction between host photoreceptors and bipolar cells; (d) host immune response to allogeneic transplants (including stem cell-derived transplants); and (e) cancer risk. In this review, the focus will be on: (a) the impact of the retinal and subretinal microenvironment in AMD eyes and (b) the impact of iatrogenic retinal detachment required for cell delivery.

Retinal and Subretinal Microenvironment

Bruch's membrane exhibits a number of abnormalities with aging and in AMD, including thickening and accumulation of abnormal extracellular material, protein crosslinking and non-collagenous protein deposition, lipidization, and deposition of inflammatory mediators [2, 3, 51–53]. In addition, RPE exhibit signs of senescence, and areas of choriocapillaris dropout are present [54]. These changes may prevent transplanted RPE from surviving and differentiating in AMD eyes [55].

As there are no animal models that faithfully reproduce all aspects of AMD pathology [56], we have studied RPE transplant survival in organ culture using post-mortem human AMD eyes to analyze RPE-Bruch's membrane interactions that may occur during RPE transplants in AMD patients. Data from post-mortem human eyes indicate that RPE transplant survival and differentiation on untreated aged and AMD Bruch's membrane is poor [57]. The results observed in organ culture mimic closely those observed in transplants of RPE suspensions in humans with GA [33, 58]. Furthermore, we found that in organ culture, survival and differentiation of human RPE suspensions in areas of GA could be improved substantially by incubating the cells in bovine corneal endothelial cell-conditioned medium [58]. Human ESC-RPE survival as well as fetal human RPE survival increased 400% on aged and AMD Bruch's membrane in the presence of this conditioned medium (Figs. 1, 2). In these experiments, the organ culture medium is changed three times per week as the volume of conditioned medium is low (200 µl), and it tends to evaporate. In addition, the conditioned medium vehicle for these experiments is Madin-Darby Bovine Kidney Maintenance Medium (MDBK-MM; Sigma–Aldrich, St. Louis, MO) supplemented with 2.5 µg/ml amphotericin B and 50 µg/ml gentamicin. MDBK-MM is a serum- and protein-free, defined medium designed for maintaining high-density cultures of MDBK cells. Providing a serum-containing vehicle does not materially improve RPE survival on aged human Bruch's membrane [58]. We have found that unless the cells are exposed to the conditioned medium throughout 21 days in organ culture, RPE survival is not optimal [58]. This result may mean that continuous delivery of the bioactive components may be needed for an extended period of time in situ.

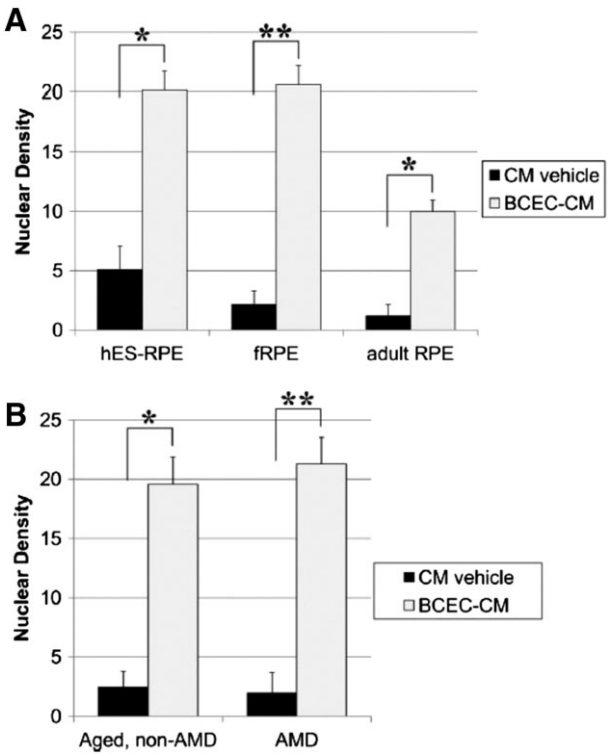

Figure 1. Nuclear densities of cells seeded on aged submacular Bruch's membrane explants after 21-day culture in conditioned media (CM) vehicle or bovine corneal endothelial cell (BCEC)-CM (paired explants from the same donor). **(A):** Nuclear density comparison of retinal pigment epithelium (RPE) cells derived from hESC-RPE (n = 6), cultured human fetal RPE (fRPE, n = 22), and cultured human adult RPE (donor ages 58, 71, and 78 years; n = 7). Within each group, significant differences were observed between cells cultured in CM vehicle and cells cultured in BCEC-CM. The nuclear density of cells cultured in CM vehicle was not statistically different between groups. The nuclear densities of hES-RPE and fRPE were not significantly different from each other but were significantly higher than the nuclear density of adult RPE cells after culture in BCEC-CM. **(B):** Comparison of nuclear densities of fRPE on age-matched, non-AMD versus AMD Bruch's membrane at day 21. Explants seeded with fRPE on aged Bruch's membrane (n = 9) were compared with explants seeded on age-related macular degeneration (AMD) submacular Bruch's membrane (n = 13). No significant differences were observed in the nuclear densities of fRPE on non-AMD versus AMD explants for a given medium, although the nuclear density was significantly higher in the presence of BCEC-CM versus CM vehicle. Nuclear density values are counts of nuclei of cells directly in contact with Bruch's membrane, expressed as mean nuclear density per millimeter Bruch's membrane. Bars indicate mean ± SE nuclear density. *, $p < .05$; **, $p < .001$. Reproduced with permission from Sugino et al. [55].

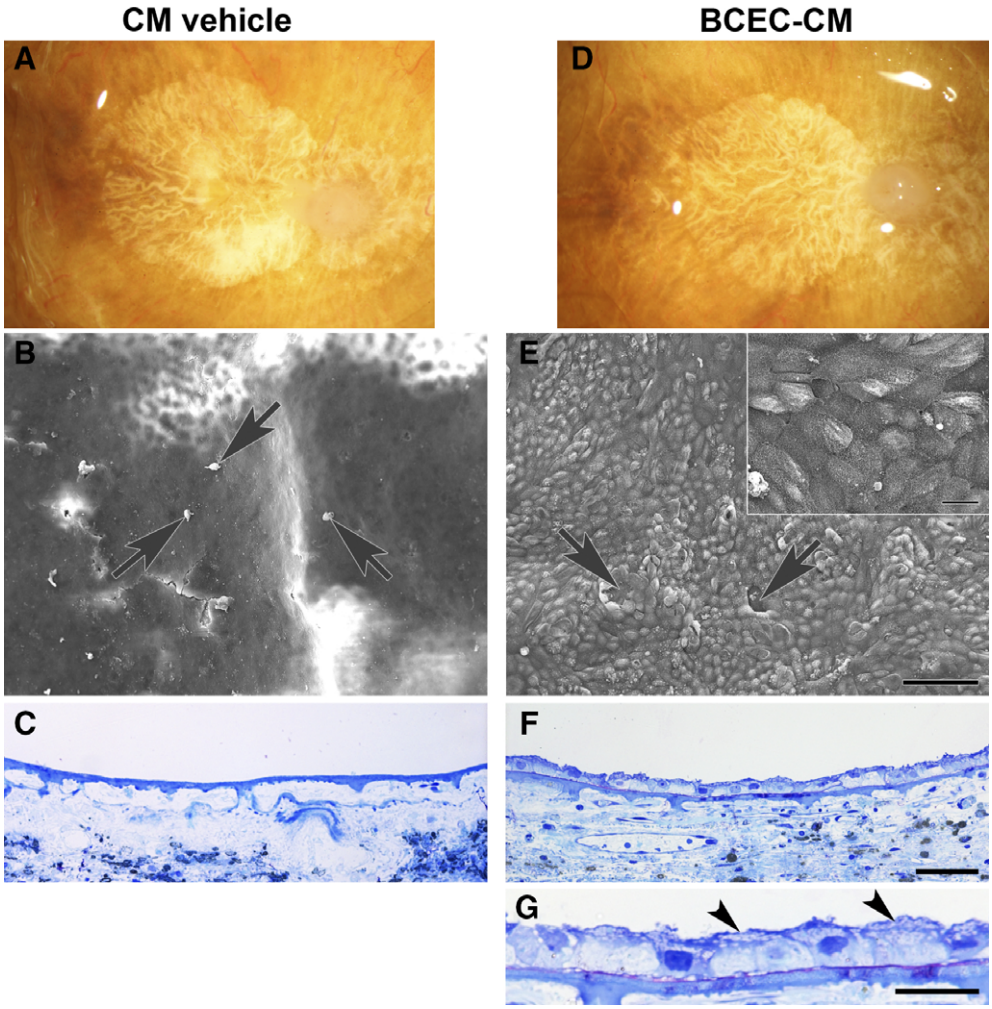

Figure 2. Paired explants from an 82-year-old woman with geographic atrophy, seeded with fetal retinal pigment epithelium (RPE) cells. The patient's clinical history noted age-related macular degeneration (AMD) for 20 years. **(A, D):** Post-mortem clinical photographs showing subfoveal geographic atrophy before RPE cell seeding. In conditioned media (CM) vehicle, **(B)** only a few dead cells (arrows) and cellular debris are present on the explant surface. **(C):** No cells are present on Bruch's membrane surface. In bovine corneal endothelial cell (BCEC)-CM, **(E)** RPE cells fully resurface Bruch's membrane in the area of geographic atrophy with a few very small defects (arrows). Localized areas of multilayering are present. Cell surfaces show abundant apical processes (inset). **(F):** In this field, cells resurfacing the BCEC-CM explant are predominantly bilayered. Cells directly on Bruch's membrane are small and tightly packed; flat cells appear to overlie the cells in contact with Bruch's membrane. **(G):** Flattened cell processes overlying cells on top of Bruch's membrane are indicated by arrowheads. The cell processes contain vesicles. CM vehicle nuclear density (ND), 0; BCEC-CM ND, 19.61 $\pm$ 0.43. Scale bars: 100 µm (E); 20 µm (E, inset); 50 µm (F); 20 µm (G). Toluidine blue staining. Reproduced with permission from Sugino et al. [58].

All the RPE survival-related biological activity of this conditioned medium is present in a fraction that contains components filtered through a 50 kDa molecular weight (MW) cutoff filter [59]. In fact, two MW filtrates (a low MW fraction collected from a 3 kDa cutoff filter and a 10–50 kDa filtrate collected from 50 kDa filtrates further purified with a 10 kDa cutoff filter) when combined are sufficient for the full biological effect of the conditioned medium. The identity of the bioactive components is under investigation, but the important point is that one may be able to improve the survival and differentiation of RPE suspensions in areas of GA without using a scaffold. Scaffolds have a number of potential advantages: (a) one can deliver a monolayer of differentiated RPE, which may promote more rapid and effective photoreceptor rescue than a cell solution; (b) the scaffold prevents transplanted RPE from interacting with aged/damaged Bruch's membrane, which may contain death signals (or their equivalent) [60]; and (c) RPE on scaffolds may be more resistant to oxidative damage than RPE suspensions [61, 62]. Scaffolds are associated, however, with some limitations. First, the size of the retinotomy required to deliver the cells is considerably greater (10–20×) than with cell suspensions, which creates a greater risk for epiretinal membrane formation and postoperative retinal detachment; 38- to 41-gauge retinotomies are essentially self-sealing whereas the retinotomies used to deliver scaffolds currently require a retinal incision of a size that is best treated with laser photocoagulation to prevent postoperative retinal detachment. Second, the surgical procedure for subretinal scaffold delivery is more complex than injection of a cell suspension; it requires special instruments and involves

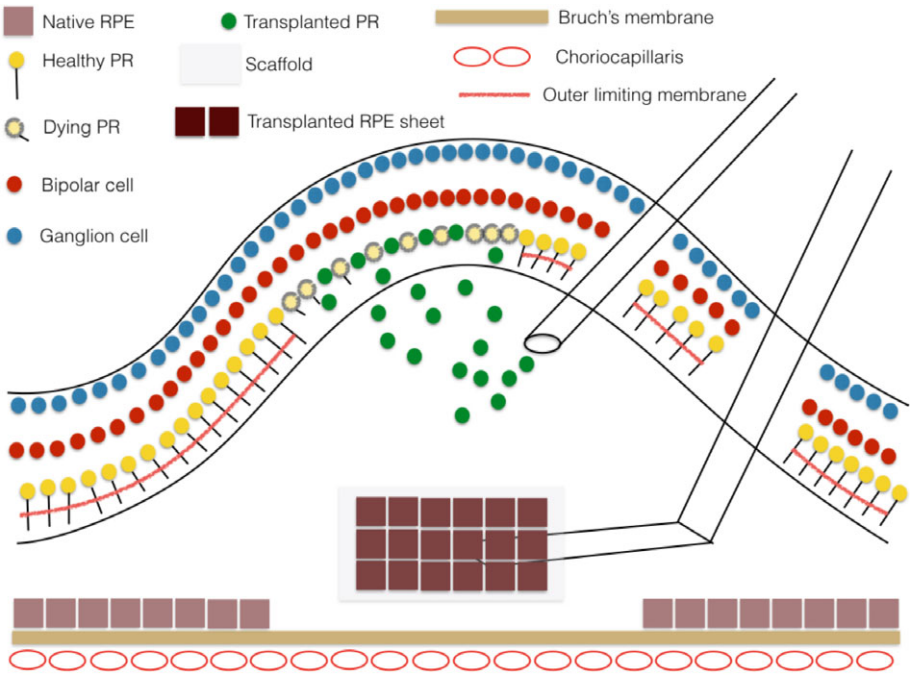

Figure 3. Schematic drawing illustrating subretinal injection of a suspension of rod photoreceptor precursor cells as might be done for a patient with photoreceptor degeneration due to a retinal dystrophy. The cells integrate into the retina preferentially in areas of external limiting membrane breakdown. Also shown is subretinal delivery of a retinal pigment epithelium (RPE) sheet on a scaffold to replace a localized RPE defect on Bruch's membrane as could occur in patients with geographic atrophy. Cell delivery to the subretinal space requires creating a localized retinal detachment. Reproduced with permission from Zarbin [15].

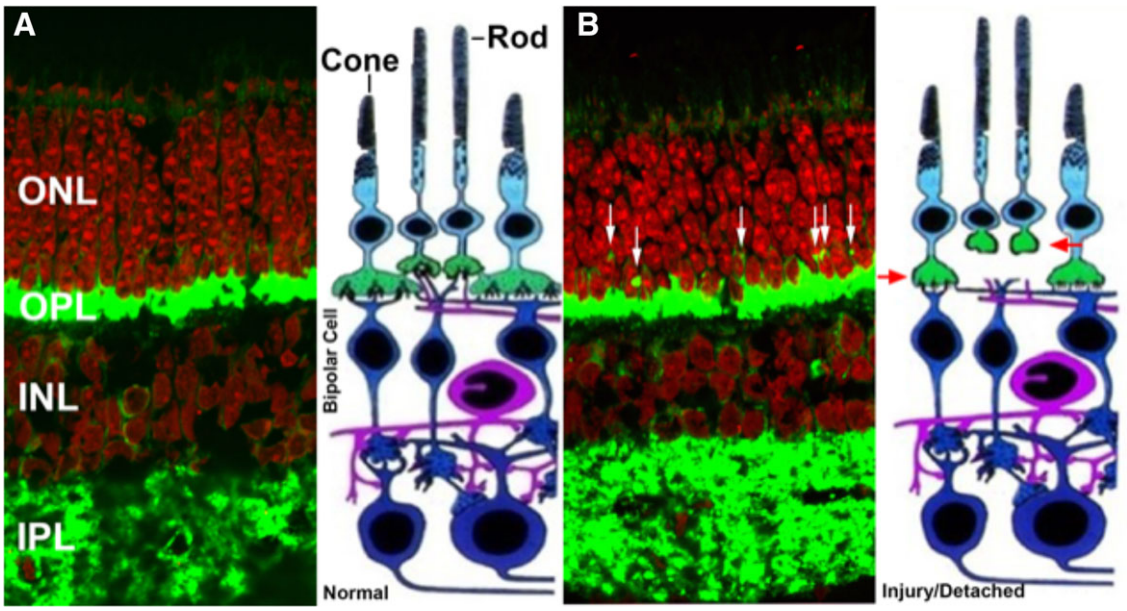

Figure 4. Injury-induced synaptic disjunction. **(A):** Normal retina labeled for synaptic protein (SV2, green) and nuclei (red). **(B):** After detachment, rod terminals retract from the outer plexiform layer into the outer nuclear layer (white arrows) and cone terminals either round up or flatten due to reduction in invaginations in the cone terminals (red arrows, injured cone and rod terminals). Cone terminals are not readily evident in the photomicrograph (visualized as a reduction in invaginations in the cone terminals) due to the paucity of cones in this region but are illustrated in the adjacent schematic drawing. Abbreviations: INL, inner nuclear layer; IPL inner plexiform layer.

creating a larger area of retinal detachment to deliver the scaffold to the subretinal space. Considering the number of patients that would require cell therapy, if it were highly effective, the greater ease of skill acquisition associated with delivery of cell suspensions may present a considerable advantage with regard to adoption of treatment by practicing retina surgeons. Finally, molecules that improve RPE survival as a differentiated monolayer on AMD Bruch's membrane can also be

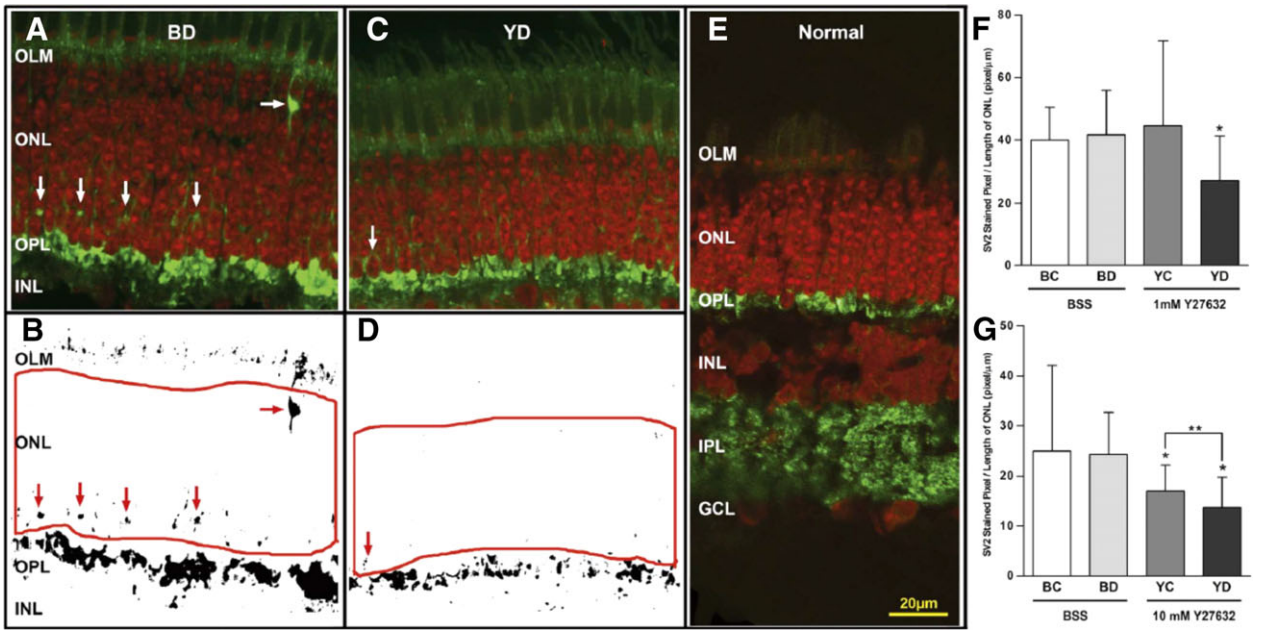

Figure 5. Effect of the ROCK inhibitor, Y27632. On axonal retraction by photoreceptors after retinal detachment in vivo. **(A, C):** Representative confocal images of control detached retina (BD) and detached retina treated with 10 mM Y27632 (YD) labeled for SV2 (green) and nuclei (red). SV2 labels rod presynaptic terminals. SV2-labeled spots (white arrows) in the outer nuclear layer (ONL) indicate axonal retraction. **(B, D):** Binary images created from (A) and (C). SV2-labeled spots are indicated with red arrows. The number of labeled pixels in the ONL delimited by the red borders was determined and divided by the length of examined ONL. **(E):** Normal retina without detachment is shown for comparison. **(F, G):** Treatment with 1 mM and 10 mM Y27632. Comparison of SV2-labeled pixels per unit retinal length in different retinal areas (BC and BD, attached and detached areas, respectively, in the eye using balanced salt solution [BSS] for detachment; YC and YD, attached and detached areas, respectively, in the eye using Y27632 for detachment). In both treatment groups, there are no significant differences between BC and BD in SV2-labeled pixels. For 1 mM Y27632, labeled pixels in YD were 34.5% less than labeled pixels in BD (*, p = .02; n = 48 retinal sections; 16 samples; four pigs). There was also a reduction in pixel labeling to 38.7% in YD compared with that in YC (p = .06; n = 48 retinal sections; 16 samples; four pigs). For 10 mM Y27632, labeled pixels in YD and YC were 43.7% (*, p = .02) and 29.9% (*, p = .04), respectively, less than labeled pixels in BD. Thus, only with 10 mM Y27632 was there a reduction in pixel labeling in the YC area compared with that in BD. Finally, there was also a significant reduction in labeled pixels in YD compared with that in YC by 24.4% (**, p = .009; n = 48 retinal sections; 16 samples; four pigs). Reproduced with permission from Wang et al. [71].

incorporated into scaffold technology, which might improve long-term RPE survival in situ when using this approach to cell delivery.

Cell Delivery Via Retinal Detachment

All methods of RPE transplantation require creation of a retinal detachment so that cells can be delivered to the space between Bruch's membrane and the neural retina (Fig. 3). Retinal detachment affects synapses in the outer plexiform layer [63, 64]. Synaptic injury begins with retraction of rod presynaptic terminals toward their cell bodies (Fig. 4). Axonal retraction results in disjunction of the first synapse in the visual pathway as the rod presynaptic terminal disconnects from the postsynaptic bipolar cell dendrite [65]. Retinal detachment also disrupts cone terminals, which lose their synaptic invaginations and normal connections with bipolar cells [64, 66]. Shortly after rod terminal retraction, bipolar cell dendrites extend into the outer nuclear layer, and horizontal cells exhibit sprouting and sometimes extend into the subretinal space [64]. Rod axon retraction and bipolar and horizontal cell sprouting occur in humans after retinal detachment [66, 67]. Retinal detachment is also associated with some degree of neuronal apoptosis even if the detachment is relatively brief. Retinal reattachment allows photoreceptor outer segments to regrow but does not restore retinal synaptic structure completely [68, 69].

Fontainhas and Townes-Anderson showed that synaptic retraction is associated with a significant increase in RhoA-GTP formation, and this biochemical change begins within minutes after retinal injury or detachment [70]. Synaptic retraction can be blocked using Rho A antagonists [70]. RhoA activates Rho-associated protein kinase (ROCK). ROCK belongs to the AGC (PKA/PKG/PKC) family of serine-threonine kinases. ROCK is involved mainly in regulating the shape and movement of cells by acting on the cytoskeleton. Through its actions on LIM kinase and cofilin, ROCK increases actin depolymerization. In addition, by phosphorylating myosin light chain, ROCK induces actin binding to myosin II and contractility increases. Human ROCK1 is a major downstream effector of the small GTPase RhoA. Thus, one can block the effects of RhoA with ROCK inhibitors.

Using an in vivo model of retinal detachment in pigs, Wang et al. found that rod-bipolar synaptic disjunction not only occurs in the area of the detachment, it also occurs in attached retina millimeters away from the area of detachment (Fig. 5) [71]. The implication of this finding is that even if surgeons attempt to spare the fovea from detachment when delivering drugs, cells, or genes to the subretinal space, an extrafoveal detachment in the macular area is likely to induce synaptic changes in the fovea that may compromise the patient's final vision. In addition,

Wang et al. found that rod-bipolar synaptic disjunction could be reduced with subretinal administration of a ROCK inhibitor (Fig. 5) [71]. Townes-Anderson et al. reported that subretinal injection of fasudil, a ROCK inhibitor that is approved for clinical use for a different indication, also reduces rod-bipolar synaptic disjunction [72]. Intravitreal injection is also effective, which may facilitate clinical use, as intravitreal injections are done routinely in an outpatient setting in most retina clinics. Another benefit of ROCK inhibition is that it reduces photoreceptor apoptosis induced by retinal detachment [72].

Conclusion

Cell therapy can have the objectives of rescue (i.e., modulation of metabolic abnormalities primarily for sight preservation) as well as replacement (i.e., replace cells lost due to injury or disease with the goal of sight restoration as well as preservation). The first clinical trials of RPE transplantation for the late complications of AMD have begun with some preliminary signs of success (e.g., improvement in vision in some patients, anatomic evidence of transplant-host integration with some evidence of host photoreceptor recovery, long-term survival of autologous iPSC transplants without immune suppression) as well as limitations (e.g., limited RPE suspension survival in the AMD eye, limited tolerance for long-term systemic immune suppression in elderly patients, suggestion of uncontrolled cell proliferation in the vitreous cavity). RPE survival on aged and AMD Bruch's membrane can be improved with chemical treatment. This finding establishes the possibility that RPE transplants may survive in AMD eyes without the use of a scaffold, which, if true, may enhance the efficacy of transplants of RPE suspensions in AMD eyes. Delivery of cell suspensions is technically easier and possibly safer than delivery of cells on a scaffold. Nonetheless, these bioactive moieties might also be integrated into scaffolds used to deliver cells to the subretinal space. Retinal detachment, currently used to deliver transplanted RPE cells, induces disjunction of the first synapse in the visual pathway: the photoreceptor-bipolar synapse. This synaptic change occurs even in areas of attached retina near the locus of detachment. Synaptic disjunction and photoreceptor apoptosis associated with retinal detachment can be reduced with Rho kinase inhibitors. Addition of Rho kinase inhibitors may improve retinal function and photoreceptor survival after subretinal delivery of cells either in suspension or on scaffolds.

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Author Contributions

M.Z.: conception, preparation of manuscript, generation of some data presented in manuscript; I.S., E.T.-A.: revision of manuscript, generation of some data presented in manuscript.

Disclosure of Potential Conflicts of Interest

Dr. Zarbin is a paid consultant for Cell Cure, Chengdu Kanghong Biotechnology Co., Coherus Biosciences, Inc., Daiichi Sankyo, Frequency Therapeutics, Genentech/Roche, Healios KK, Inc., Iridex, Isarna Therapeutics, Makindus, Novartis Pharma AG, Ophthotech Corp., and Percept Corp. Work on improving RPE survival on AMD Bruch's membrane is covered in U.S. Patent no. 9598672, issued March 21, 2017 ("Production of extracellular matrix, conditioned media and uses thereof." Inventors: Sugino I (Madison, NJ), Zarbin, M. (Chatham, NJ), Sun Q. (West Orange, NJ), Birge R. (New York, NY). The other authors indicated no potential conflicts of interest.

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