Chapter 1
The Eye as a Target Organ for Stem Cell Therapy

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Abstract Retinal degenerations are a heterogeneous group of disorders that are characterized by progressive cellular dysfunction, cellular disarray, and eventually cell death. Early in the course of disease therapeutic intervention consists of pharmaceutical treatment to prevent cell death or gene therapy to correct the underlying mutation. Due to the nature of pathologies involving these disorders, particularly in late stage of disease, cell replacement therapy or electric stimulation of remaining cells by artificial retinal prosthesis is the only viable option. Stem cell therapies for retinal degenerative diseases such as age-related macular degeneration (AMD) and retinitis pigmentosa (RP) are a promising therapeutic option and will require replacement of lost photoreceptor cells and retinal pigment epithelium (RPE). Current clinical trials are underway to evaluate the potential of stem cell therapy in humans. The use of induced pluripotent stem (iPS) cells hold great promise as a potential reservoir of cells for the treatment of retinal disorders as well as a clinical tool to help understand disease pathology. Advances in stem cell technology will translate these therapies into viable clinical options for the treatment of retinal degenerative diseases and other disorders.

Introduction

Retinal degenerations are a heterogeneous group of disorders that are characterized by progressive cellular dysfunction, cellular disarray, and eventually cell death. Numerous classification systems exist for these disorders, but no one classification

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system captures the complexity of the disease processes, the diversity of their pathology, and the common themes in treatment that underlie these diseases. Many current classifications distinguish between macular diseases and peripheral retinal degenerations, but this classification system does not represent the complexity of the disease process in a complete fashion. Prior to the discovery of gene mutations that increase the risk profile for age-related macular degeneration (AMD), retinal degenerations were often classified as either hereditary or nonhereditary diseases, but the simplicity of this classification has been called into question based on the observation that certain alleles increased the risk of AMD [1–4]. Thus, for the purpose of this discussion, retinal degenerations will be classified by whether they are Mendelian disorders (e.g., most if not all forms of retinitis pigmentosa (RP), Leber's congenital amaurosis, and Best's disease) or non-Mendelian retinal disorders, including AMD.

Because of the complexity of the disease processes, it is possible to dedicate an entire chapter of this book to each disease and still not cover all the details of each condition. However, regardless of the cause of the retinal disorder, it is important to recognize that severe vision loss is typically associated with cellular dysfunction or death. Early in the course of many diseases there is cell dysfunction without cell death. In these early stages, gene therapy, pharmacological treatment to manipulate the cell death pathway, and/or treatment with locally administered growth factors, such as ciliary neurotrophic growth factor, may all prove to be useful. However, late stages of retinal disease, which are usually accompanied by severe vision loss, will require a different approach. For example, in advanced stages of many forms of RP, severe vision loss is due to death of photoreceptors, loss of the native retinal pigment epithelium (RPE) monolayer on Bruch's membrane, migration of pigmented cells into the retina, and transsynaptic degeneration leading to inner retinal disturbance. In advanced geographic atrophy in AMD, there is loss of RPE and photoreceptors and secondary atrophy of the choriocapillaris. Reversal of vision loss in these late stages of disease, after cell loss has occurred, will likely require cell therapy with transplantation of photoreceptors, RPE and/or choriocapillaris cells; or direct electrical stimulation of the inner neural retina with multi-electrode arrays.

In this review we will discuss the clinical and pathological features of retinal degenerations that are important to their potential treatment with stem cell therapy; the unique combination of eye anatomy and imaging capabilities that makes it an excellent target organ for early stem cell therapy in humans; and the status of human trials.

Clinical and Pathological Features of Retinal Degenerations

Retinitis Pigmentosa

Retinitis pigmentosa (RP) is a group of Mendelian hereditary disorders characterized clinically by bilateral progressive loss of peripheral vision, a marked ring-like constriction of the visual field, night blindness, and late loss of central

vision. As a group the population prevalence of RP is about 1:4,000, so the estimates are that approximately 100,000 in the USA have this disease. Investigators have identified at least 45 loci for mutations that can cause retinitis pigmentosa, and these genes collectively account for disease in a little over half of all patients [5–7]. Of the cloned genes for retinitis pigmentosa it is estimated that dominant retinitis pigmentosa account for about 50 %, recessive retinitis pigmentosa account for about 40 % and X-linked retinitis pigmentosa account for approximately 80 % of cases, indicating that many genes remain to be identified [6, 8]. Rods are the predominantly affected photoreceptors and dysfunction causes night blindness and peripheral field loss beginning as early as the teenage years [9]. Disease progression leads to central acuity loss and legal blindness in the majority of patients [10]. Classic findings on funduscopic exam include perivascular bony spicule pigmentation, attenuated arterioles, and waxy optic disc pallor, typically associated with vitreous cells and posterior subcapsular cataracts. However, many of these findings may be absent in early stages of disease [11, 12]. Electroretinogram (ERG) testing is important for diagnosis and may provide prognostic information [10]. The genetics of retinitis pigmentosa are extremely complex with diverse modes of inheritance [12]. Potential interventions include vitamin A therapy and carbonic anhydrase inhibitors, but treatment options are extremely limited in the majority of cases with no effective form of therapy. Results evaluating vitamin A efficacy have shown limited benefit but potential risks exist with oral vitamin A supplementation, including the risk of hepatotoxicity [13]. Carbonic anhydrase inhibitors have shown clinical benefit in reducing macular edema and improving visual acuity in some patients with retinitis pigmentosa [14].

Genetics

The genetics of retinitis pigmentosa are extremely complex with diverse modes of inheritance including dominant, recessive, X-linked, mitochondrial, and digenic forms [12]. The disease may manifest solely with visual symptoms or may be accompanied by a constellation of systemic findings in patients with syndromic retinitis pigmentosa. The diversity in genetic transmission and clinical presentation is not entirely surprising given that retinitis pigmentosa constitutes a broad group of diseases that arises from diverse biological pathways.

Retinitis pigmentosa demonstrates multiple modes of segregation [15]. Autosomal dominant transmission occurs most frequently and accounts for 20 % of retinitis pigmentosa cases. Symptoms are generally less severe with adult-onset with variable penetrance of symptoms. Autosomal recessive disease occurs in 13 % of cases and is characterized by earlier onset of symptoms and severe vision loss. X-linked recessive disease accounts for 8 % of cases and has the poorest visual prognosis with early onset and rapid progression of symptoms [12]. Visual deficits typically present within the first decade of life and progress to partial or complete blindness by the third or fourth decade. In approximately 20 % of nonsyndromic cases, the mode of

transmission cannot be established because of an unclear family history. These cases are termed simplex retinitis pigmentosa and presumed to arise from autosomal recessive or X-linked transmission. Syndromic retinitis pigmentosa, in which vision loss occurs in the settings of extraocular disease manifestations, constitutes 25 % of cases with Usher (10 %) and Bardet–Biedl (5 %) syndromes occurring most frequently [15].

Mutations in 53 genes are known to cause nonsyndromic retinitis pigmentosa or Leber's congenital amaurosis (LCA), which may be indistinguishable from early onset retinitis pigmentosa. This includes 25 genes in autosomal recessive retinitis pigmentosa (arRP), 17 genes in autosomal dominant retinitis pigmentosa (adRP), 13 genes in recessive LCA, 2 genes in dominant LCA, and 6 gene mutations in X-linked retinitis pigmentosa (xlRP) [15]. Mutations in a single gene, such as rhodopsin or neural retina-specific leucine zipper (NRL), may result in multiple forms of disease such as adRP and arRP. The proportion of disease caused by mutations in a particular gene is highly variable [15]. The largest proportion of retinitis pigmentosa is caused by mutations in rhodopsin (RHO) in adRP (26.5 %), Usher syndrome 2A (USH2A) in arRP (10.0 %), retinal guanylate cyclase 2D (GUCY2D) in recessive LCA (21.2 %), and retinitis pigmentosa GTPase regulator (RPGR) in xlRP (74.2 %). A significant proportion of the molecular defects underlying retinitis pigmentosa are known to affect the phototransduction cascade, visual cycle, outer segment structure, cilium-mediated protein trafficking, cellular interaction/adhesion, transcription factors, and RNA-intron splicing factors.

Symptoms and Clinical Findings

Retinitis pigmentosa is phenotypically heterogeneous with wide variation in severity, age of onset, and progression. Classically, retinitis pigmentosa manifests with early night blindness (nyctalopia) beginning in teenage years followed by loss of peripheral visual field. The majority of patients are classified as legally blind by age 60 with central visual field diameters less than 20° [9]. Defects in blue–yellow color perception may occur in advanced stages when visual acuity is 20/40 or worse [16].

Syndromic retinitis pigmentosa is a term used to describe cases of retinitis pigmentosa associated with extraocular symptoms. Approximately 25 % of retinitis pigmentosa cases are syndromic and over 30 forms have been identified [17]. Usher syndrome is the most common form and is associated with sensorineural deafness. It accounts for about 10 % of retinitis pigmentosa cases and is divided into three major groups. Type 1 demonstrates profound congenital deafness, vestibular symptoms, and childhood-onset retinopathy [18]. Type 2 manifests with congenital partial, nonprogressive deafness, absence of vestibular symptoms, and mild later-onset retinopathy [19, 20]. Type 3, the least common form, demonstrates progressive deafness beginning in the third decade and adult-onset retinopathy [21]. Bardet–Biedl syndrome is the second most common form of syndromic retinitis

pigmentosa and accounts for 5 % of retinitis pigmentosa cases [15]. It is associated with polydactyly, obesity, renal dysfunction, and mental retardation. Other forms of syndromic retinitis pigmentosa account for 10 % of all retinitis pigmentosa cases and include Refsum's disease, Bassen–Kornzweig syndrome, Kearne–Sayre syndrome, Batten's disease, and Senior–Loken disease. A complete listing of genes implicated in retinitis pigmentosa can be found on the Retinal Information Network web site http://www.sph.uth.tmc.edu/retnet/.

Retinitis pigmentosa classically leads to fundus changes with accumulation of bony spicule pigmentation. Lesions are generally perivascular and localized to the mid-periphery where rods are concentrated. However, pigment distribution is often variable and may be diffuse, sectoral, or even be absent in certain subtypes of retinitis pigmentosa. Other signs include abnormal retinal pigmentation changes, attenuated arterioles, vitreous cells, waxy optic disc pallor, and blue–yellow color vision deficiency. Vitreous cells and opacities are the most consistent characteristics across all forms of retinitis pigmentosa. Notably, early stages of retinitis pigmentosa may lack appreciable funduscopic findings [5, 11, 12]. Retinitis pigmentosa patients, particularly those over age 40, may demonstrate cystoid macular edema, epiretinal membranes, diffuse retinal vascular leakage, macular preretinal fibrosis, macular RPE defects, and posterior subcapsular cataracts. Other associated findings include myopia and astigmatism [5, 11, 12, 22–24].

Treatment

Treatment options are extremely limited for most retinitis pigmentosa subtypes with no effective approach for prevention, stabilization, or reversal of visual loss.

The efficacy of vitamin A and E supplements on slowing retinitis pigmentosa progression was examined in a randomized, double-masked, prospective study [13]. About 601 patients with non-syndromic retinitis pigmentosa and Usher syndrome (type 2) were randomized into four treatment groups receiving 15,000 IU/d of vitamin A, 400 IU/d of vitamin E, 15,000 IU/d of vitamin A plus 400 IU/d of vitamin E, or trace amounts of both vitamins and followed for 4–6 years. The trial concluded that (1) vitamin A groups demonstrated slower rates of decline in cone ERG amplitudes (2) vitamin A groups were 32 % less likely to have a decline in ERG amplitude of 50 % or more from baseline (3) vitamin E groups were 42 % more likely to have a decline in ERG amplitude of 50 % or more from baseline, and (4) there was no significant difference in visual acuity and field loss. The reduction of ERG decline in patients receiving vitamin A was limited to the 30 Hz and 0.5 Hz flash amplitudes. Significantly, these patients did not demonstrate any improvement in psychophysical visual parameters [25, 26].

Thus, these results suggest that benefits of vitamin A therapy are limited and must be weighed against potential risks such as teratogenic effects in pregnant women, elevated intracranial pressure, hepatomegaly, bone disease in young individuals, and elevated serum lipids [27–29]. Currently many practitioners do

not use vitamin A supplementation routinely due to the small treatment effect and the need for monitoring of vitamin A toxicity. In addition, the mixed molecular etiology of retinitis pigmentosa suggests that response to vitamin A may vary across retinitis pigmentosa subtypes. Studies in ABCA4 knockout mice demonstrated increased rates of lipofuscin deposition and photoreceptor degeneration in mice on vitamin A supplementation. These results suggest that if vitamin A supplementation is employed, it should be done so selectively [30, 31] as it may have a deleterious effect on certain subsets of retinitis pigmentosa patients. Because of the small magnitude of the effect on ERG, lack of improvement in psychophysical parameters, concerns about toxicity, and the varied genetics of retinitis pigmentosa, the use of vitamin A supplementation to slow retinitis pigmentosa progression has not been universally adopted. If patients are placed on oral vitamin A therapy, they should undergo periodic liver function testing, osteoporosis screening, and fasting serum vitamin A measurements to avoid toxicity.

Other therapies have also been advocated as potentially effective in retinitis pigmentosa. To date, however, there is no evidence of clinical visual improvement with lutein supplements [32], docosahexaenoic acid supplements [33–35], light deprivation [36], therapeutic bee stings [37], vasodilators [38], or placental tissue injections [39]. Interestingly, repeat intravitreal injections and/or pars plana vitrectomy are not currently used to treat patients with retinal degenerations, despite the fact that there is a well-known rescue effect of vitreous and subretinal surgery on retinal degeneration. Subretinal insertion of a dry needle results in a degree of photoreceptor rescue similar to that of intravitreal or subretinal basic fibroblast growth factor injection in the Royal College of Surgeons rat [40]. Anterior chamber injection of placebo and brain-derived neurotrophic growth factor produces similar rescue effects in axotomized rat ganglion cells [41]. Lensectomy and vitrectomy alone rescue degenerating photoreceptors in the P347L transgenic pig, which contains a rhodopsin mutation known to cause retinitis pigmentosa in humans [42]. Subretinal saline injection produces a rescue effect in the Royal College of Surgeons rat [43]. These studies demonstrate clearly that vitreous and subretinal surgery alone may produce some rescue effect in retinal degenerations, but long-term demonstration of their efficacy awaits additional preclinical and clinical trials.

There is some therapeutic benefit of dietary modifications and nutritional supplements for two rare forms of syndromic retinitis pigmentosa. Phytanic acid oxidase deficiency (Refsum's disease) arises from failure of phytanic acid degradation and consequent elevation of serum phytanic acid. Clinical manifestations include ataxia, peripheral neuropathy, deafness, and cardiac conduction defects [44–46]. Dietary restriction of phytanic acid may halt or reduce progression of retinitis pigmentosa. Abetalipoproteinemia (Bassen–Kornzweig syndrome) is characterized by low serum levels of apolipoprotein B, resulting in fat malabsorption and low plasma concentrations of fat-soluble vitamins. Systemic signs generally manifest in childhood and include diarrhea, cerebellar ataxia, and acanthocytosis. Therapy with high doses of vitamin A may allow rapid restoration of visual function in early stages of disease [47–49]. Laboratory studies of serum phytanic acid levels and serum lipoprotein electrophoresis can assist in the diagnosis of Refsum's disease and Bassen–Kornzweig syndrome, respectively.

Visual function in retinitis pigmentosa may be improved by monitoring for and treating associated conditions such as cystoid macular edema, posterior subcapsular cataract, and epiretinal membranes. In addition, referral to low vision clinics can help optimize remaining visual function.

The Argus II Retinal Prosthesis System developed by Second Sight Medical Products, Inc. is intended to provide electrical stimulation of the retina to elicit visual perception in blind subjects with retinitis pigmentosa [50]. The technology is currently being evaluated in a clinical study conducted in the USA and recently received a CE (European Conformity) mark in Europe which is a key indicator of a product's compliance with European Union legislation. The device consists of a surgically implanted 60-electrode stimulating microelectrode array consisting of 200 μm diameter disc electrodes, an inductive coil link used to transmit power and data to the internal portion of the implant, an external belt-worn video processing unit and a miniature camera mounted on a pair of glasses [51, 52]. The video camera is designed to capture a portion of the visual field and relay the information to the video processing unit. The video processing unit then digitizes the signal in real time, applies a series of image processing filters, down-samples the image to a 6×10 pixelized grid, and creates a series of stimulus pulses based on pixel brightness values and look-up tables customized for each subject [51]. The stimulus pulses are delivered to the microelectrode array via application-specific circuitry and a superior-temporally placed inductive radio frequency coil link allowing for wireless forward and reverse telemetry between intra and extra-ocular portions of the system [51]. The prosthesis is expected to generate limited amounts of vision in patients with severe to profound vision loss in the range of hand motions or light perception vision.

Age-Related Macular Degeneration

Age-related macular degeneration (AMD) affects 30–50 million elderly people worldwide and is the leading cause of blindness in individuals over the age of 50 in the Western world [53, 54]. It is estimated that approximately 30 % of adults over the age of 75 have some signs of AMD and that at least 10 % develop the advanced or late stage of disease [55, 56]. AMD as a disease entity primarily exists in two forms, nonexudative (atrophic or dry) AMD and exudative (neovascular or wet) AMD. Although the vast majority of patients with AMD are of the nonexudative type, approximately 90 % of significant vision loss due to AMD is secondary to central vision deterioration from the exudative type [56, 57]. Early in the course of disease there is cellular dysfunctional without cell death. In late-stage disease, AMD is characterized by extensive cell death, as with late-stage RP.

Genetics of AMD

Age-related macular degeneration is a complex disease that results from a combination of genetic and environmental factors. Many of these factors have been

identified, but some remain unknown. Because AMD occurs late in life, it has been very difficult to elucidate the genetic factors correlated with the disease. AMD's heterogenicity in phenotypes presents a challenge as well [58]. It also may be discovered that each individual's susceptibility is due to multiple genetic and environmental effects and interactions [58–62].

Symptoms and Clinical Findings

Patients with advanced AMD typically present with blurry central vision, metamorphopsia, and reduced vision. These symptoms can then evolve to a central scotoma and severe loss of vision [63]. Ophthalmoscopic examination of the fundus at late stages of disease demonstrates patchy chorioretinal atrophy in the dry type and exudation in the wet variety, often manifested by the presence of retinal hemorrhages and lipid exudate in and around the macula [63].

One of the earliest clinical findings associated with AMD is the presence of drusen, which represent accumulation of extracellular material beneath the RPE [64]. In the case of dry AMD, loss of vision develops due to loss of the RPE, photoreceptors, and/or choriocapillaris; this can lead to patches of atrophy which are manifest clinically by central and paracentral scotomas [64]. In the case of wet AMD overexpression or loss of normal apical–basal polarity in the expression of angiogenic factors such as vascular endothelial growth factor (VEGF) can cause neovascularization to arise from the neural retina (retinal angiomatous proliferation) or choriocapillaris. In early stages of the disease patients experience minimal vision loss but some symptoms may occur such as blurred vision, visual scotomas, decreased contrast sensitivity, abnormal dark adaptation, and the need for bright light or magnification to decipher images [64]. In the late stages of advanced non-neovascular disease, patients typically present with a gradual loss of vision that becomes more severe and affects central or pericentral vision [64]. This form usually progresses and leads to irreversible vision loss. In patients with neovascular disease, loss of vision can be much more sudden with loss occurring within days to weeks due to subretinal hemorrhage or fluid accumulation secondary to choroidal neovascularization [64, 65].

Treatment

While the last decade has brought about a revolution in the treatment of exudative AMD, there are currently no approved therapies for geographic atrophy. Numerous investigational therapies are in various stages of clinical trials. These include ciliary neurotrophic factor, complement inhibitors, weekly vaccination with glatiramer acetate, fenretinide and OT-551 [66–70]. These therapies are promising, but none have progressed beyond clinical trials, leaving a large void in the current therapy of geographic atrophy.

Ninety percent of AMD patients who experience severe vision loss do so as a result of choroidal or intraretinal neovascularization [71]. Choroidal neovascularization represents growth of neovascular tissue from the choriocapillaris, within Bruch's membrane, and eventually in the subretinal pigment epithelium and/or subretinal space. Retinal angiomatous proliferation is a form of wet AMD in which the abnormal vessels arise from the neural retina [72, 73]. Developing new treatments that prevent or reverse vision loss in AMD are of paramount importance due to the severe visual deficits that occur with this condition and the knowledge that disease prevalence will increase with shifting demographics of an aging western population.

Treatments for the wet form of this disease involve intravitreal antiangiogenic therapy, photocoagulation and photodynamic therapy, and vitreoretinal surgery. Intravitreal antiangiogenic treatment is currently the primary therapy for wet AMD and delivered directly to the vitreous. Treatment with intravitreal injection of anti-VEGF agents improves vision in patients with wet AMD but maintenance of the therapeutic effect requires continued administration of intravitreal agents, and this can be associated with potentially serious side effects such as endophthalmitis, retinal detachment, intraocular hemorrhage, increased intraocular pressure, and, in some cases, retinal detachment [74]. Photodynamic therapy uses light sensitive medicine that identifies abnormal vessel growth under the macula. Laser light then activates the light sensitive dye which can then decrease exudation from the neovascularization.

Despite these significant advances in the management of exudative AMD, there is a large unmet need for many patients with this condition. More than 50 % of patients do not respond to therapy with anti-VEGF drugs, and many patients with advanced disease have loss of vision due to scar formation and altered subretinal architecture. These limitations have led to the investigation of alternative treatment modalities for subfoveal exudative AMD, including subfoveal membranectomy with and without RPE transplantation or translocation [75–79] and macular translocation [80]. Initial efforts to improve vision with cell transplantation alone have not been met with success; reconstitution of the normal subretinal architecture is necessary for visual improvement in these individuals. Ultimately this will require reconstruction of macular anatomy in patients with advanced vision loss in exudative AMD [81]. Successful maculoplasty will require replacing or repairing damaged cells (using transplantation, translocation or stimulation of autologous cell proliferation); immune suppression (if allografts are used to replace damaged cells); and reconstruction or replacement of Bruch's membrane (to restore the integrity of the substrate for proper cell attachment). Successful maculoplasty will build on prior development of surgical techniques for managing severe vision loss in AMD patients with advanced subfoveal exudation. These techniques include surgical excision of choroidal neovascularization; [75–79, 82–84] surgical excision combined with allograft transplantation of adult or fetal RPE [85–96] or iris pigment epithelium [97–108] or macular translocation with or without choroidal membrane excision [109–127].

Simple excision of the subfoveal neovascular membrane in AMD leaves a large RPE defect under the fovea due to the removal of native RPE along with the surgically removed neovascular complex [128]. Resulting persistent RPE defects lead to the development of progressive choriocapillaris and photoreceptor atrophy [129]. Histopathology after subfoveal membranectomy alone shows absence of large swatches of native RPE, combined with damage to the outer retina, choriocapillaris atrophy and absence and/or damage to the inner aspects of native Bruch's membrane [130, 131]. The status of host Bruch's membrane has a profound effect on the behavior of RPE transplanted after subfoveal membranectomy [81, 132–139]. Thus reconstruction of Bruch's membrane is a necessary component for successful maculoplasty [140]. Given the issues with the status of host Bruch's membrane, and the paucity or absence of native RPE and/or photoreceptors in advanced disease states, there is a need for a combined approach with cell replacement therapy and Bruch's membrane reconstruction that will be required to reverse vision loss in these advanced disorders. There are significant logistical challenges to cell replacement therapy in this disease, including the need for large numbers of cells needed for cell replacement, and the need for immune suppression if allo grafts are used for transplantation. Transplantation of intact sheets and suspensions of primary RPE cells have been previously attempted in humans, with mixed results in terms of graft survival and improvement in vision [85, 91, 141–144]. Stem cells are an ideal replacement source for these lost or damaged cells, since stem cells have a significant ability to proliferate in vitro prior to transplantation and in vivo after subretinal transplantation.

Unique Combination of Anatomy and Imaging Capabilities that Make the Eye an Excellent Choice for Stem Cell Therapy

It is no accident that the eye has become one of the first organs to be treated with stem cell therapy in humans, as the eye is an excellent target organ for stem cell therapy [141]. There are several reasons for this, including the facts that retinal degenerations are well characterized, and excellent animal models exist for many of these diseases. In addition, the eye is optically transparent, so that the transplant site can be monitored directly with slit lamp biomicroscopy, indirect ophthalmoscopy, fundus photography, auto fluorescence imaging, fluorescein angiography, and optical coherence tomography, which gives us advantages of in vivo "histological sections" through a transplant area. In addition there is excellent function testing, including visual fields testing, multifocal electroretinogram (ERG), and microperimetry.

Autofluorescence imaging is a technique that allows for topographic mapping of fluorescence emanating from the retina, retinal pigment epithelium, and choroid in health and disease [145, 146]. In this technique fluorescence-based images of the human fundus are captured using different combinations of excitation and barrier filters, allowing the ophthalmologist to discern the topographic distribution of various fluorophores in the retina and deeper layers (Fig. 1.1). Many fluorophores
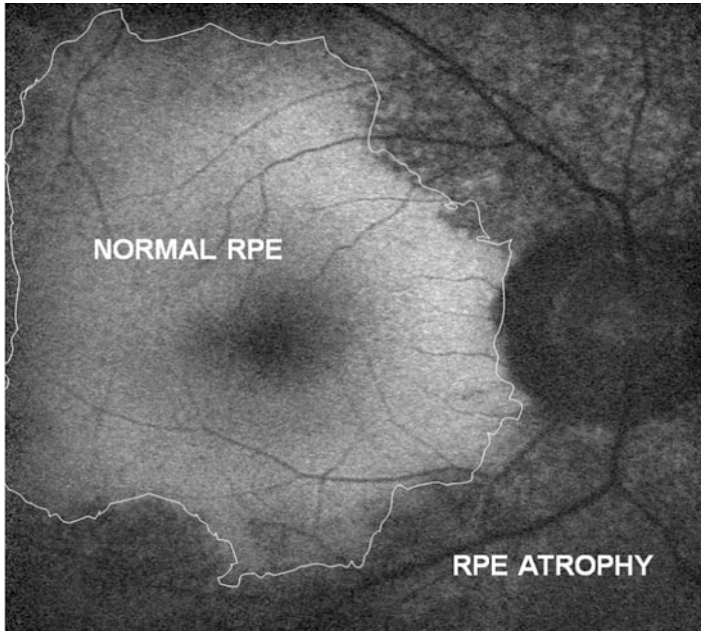
Fig. 1.1 Autofluorescence imaging of the retinal pigment epithelium (RPE), demonstrating the topographic distribution of fluorescence in the posterior segment of the human eye. Many fluorophores are contained within RPE, so that areas of normal RPE may exhibit autofluorescence compared to dark areas of patchy RPE atrophy

are contained within RPE cells, and thus the retinal pigment epithelium will fluoresce using this type of imaging, allowing for determination of areas of RPE absence and areas of atrophic patches consistent with areas of geographic atrophy (Fig. 1.1). As these techniques develop further, it is important to recognize a priori that there is no reason to think that all fluorophores will be contained within the RPE. For example, we have previously demonstrated the presence of nitro-A2E within human Bruch's membrane in elderly individuals [147], and several authors have reported decoupling of the auto fluorescent signature of A2E from the overall autofluorescent signature emanating from the human RPE. It is likely that the information obtained from autofluorescence imaging of the human retina will increase dramatically with improvement in the excitation sources and detection systems. For stem cell transplantation, autofluorescence imaging may allow us to detect the reconstruction of the RPE monolayer, if the transplanted stems cells incorporate A2E or other fluorophores after subretinal transplantation.

Fluorescein angiography (FA) is a technique used to image blood flow of the retina and choroid by using sequential fluorescence imaging following the intravenous injection of sodium fluorescein (Fig. 1.2). Histopathology studies have revealed that there is an accumulation of autofluorescent material in the retinal pigment epithelium as well as autofluorescent deposits of extracellular material in macular and retinal disease [146]. Use of fluorescein angiography allows for the
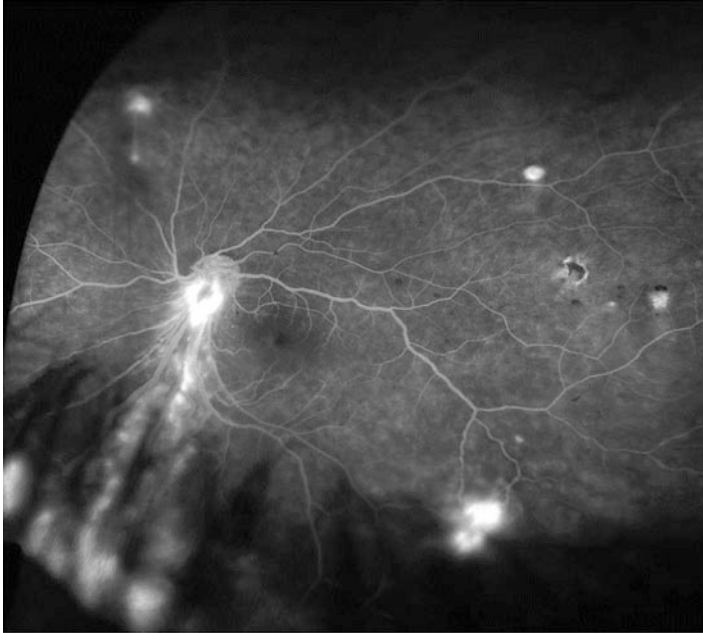
Fig. 1.2 Wide field retinal angiography to image blood flow of the retina and choroid obtained after intravenous injection of sodium fluorescein. In addition to monitoring for non-perfusion, angiography can be used to detect angioma (shown here) or vascular leakage, which can be a sign of graft rejection

visualization of atrophic patches that appear well-demarcated, hyperfluorescent areas due to loss of retinal pigment epithelium [148]. These cells, if intact, would otherwise weaken transmission.

Indocyanine green (ICG) is a cyanine dye that allows for enhanced imaging patterns of circulation when compared to fluorescein dye given a spectral absorption between 805 and 835 nm [149]. As with fluorescein dye, indocyanine allows for the visualization of atrophic areas of degeneration. Indocyanine green has primarily been used in the diagnosis and interpretation of occult choroidal neovascularization in age-related macular degeneration and for identification of angiomatous lesions of the retina and polyps in the choroid [150]. The unique properties of indocynanine green allows for visualization of macular dystrophies through overlying pathologic conditions such as hemorrhage, serous fluid, lipid, and pigment [151]. Indocyanine green has been utilized as an adjunct tool along with fluorescein angiography for the diagnosis of age-related macular degeneration [151].

Angiography allows the ophthalmologist to discern the perfusion status of the retina and choroid, as diseases that impair perfusion can be diagnosed on the basis of abnormalities in the dye filling pattern on sequential angiogram photos. In addition to monitoring for non-perfusion, FA and ICG angiography can be used

to monitor for vascular leakage, which can be diagnosed on the basis of accumulation of extracellular dye due to increased vascular leakage. There are several reasons why this is particularly useful to the field of stem cell transplantation. First and foremost, in the absence of native RPE there is secondary non-perfusion of the choriocapillaris, and this non-perfusion is evident on both fluorescein and ICG angiography. Animal studies suggest that the choriocapillaris can reperfuse after replacement of the RPE, and thus angiography can be used to monitor the outer retinal blood supply for the success of transplanted cells. Both techiques allow for the monitoring of vascular integrity (Fig. 1.2) [150, 152]. In addition ICG and FA allow the ophthalmologist to detect leakage of dye, which can be a sign of graft rejection.

Optical coherence tomography (OCT) is an imaging method currently in widespread clinical use that provides in vivo images from the human retina. OCT relies on differences in the index of refraction of ocular tissue to generate a cross-sectional image of the retina and the vitreoretinal interface. OCT can be used to measure foveal and extrafoveal retinal thickness and can be used to determine the thickness of the outer nuclear layer and integrity of the outer segment–inner segment junction. OCT can be used to detect RPE atrophy and outer retinal atrophy and to determine the thickness of the nerve fiber layer. In geographic atrophy, OCT can reveal atrophy of the choriocapillaris, particularly with enhanced depth choroid imaging. In the study done by Neurotech on ciliary neurotrophic factor (CNTF), OCT was used to demonstrate increasing thickness of the outer retina in patients with nonexudative AMD who received the CNTF-releasing implant [66]. In principle, OCT can be used to monitor the ability of transplanted stems cells to repopulate Bruch's membrane, the return of retinal thickness back to normal, and reestablishment of choroid thickness in patients with atrophy. OCT can also be used to determine adverse events after transplantation, including the development of retinal edema, after treatment of patients with exudative and nonexudative age-related macular degeneration (Fig. 1.3) [141, 153, 154].

In addition to these structural studies, there are several excellent functional tools for determining retinal function in eyes of retinal degenerations treated with stem cells. Microperimetry can assess macular sensitivity and retinal fixation by providing a retinal visual function map on a selected, localized fundus location with preset or customized scan patterns (Fig. 1.4a, b) [155]. In this technique, the retina is stimulated by illumination with small spot sizes under direct visualization; this allows the examiner to discern the retinal sensitivity as a function of illumination level and spot size in areas of the retina affected by retinal degenerations, and in treated and control regions. In principle, a beneficial effect of transplantation would be manifested by increasing retinal sensitivity on microperimetry.

Similarly, multifocal ERG can also be used to determine a decrease in retinal function due to disease, and an improvement in dysfunction after retinal transplants. Multifocal ERG allows for a topographical measure of electrical activity in distinct areas of the retina (Fig. 1.4c) [156]. Multifocal ERG can stimulate multiple retinal areas at the same time and detect each response independently [157]. In retinal
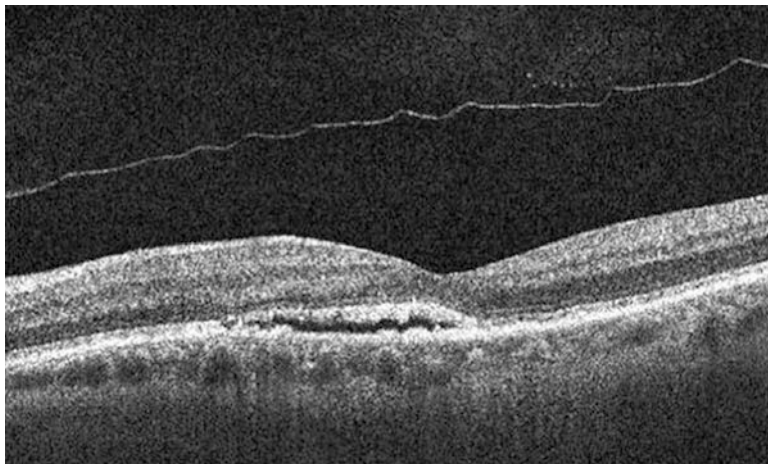
Fig. 1.3 Optical coherence tomography (OCT) reveals a small pocket of subretinal fluid in an asymptomatic patient with age-related macular degeneration. OCT can be used to measure the thickness of the outer nuclear layer and integrity of the outer segment–inner segment junction after successful cell transplantation

degenerations, there is typically a decrease in amplitude, or absent ERG signal, in areas of retinal dysfunction; this change is often present only in an area of dysfunction. There is an improvement in the global ERG in animals with retinal degenerations receiving transplants of stem cells (Fig. 1.5) [158]. In principle, improvement in retinal function after stem cell transplantation should be topographic and result in a focal improvement in ERG in the area of the transplant. These advantages in imaging and focal detection of function should not be overlooked, since cell transplants in other areas of the body do not have similar advantages. These advantages present a unique opportunity to detect the beneficial effects of stem cells in the treatment of retinal diseases. Their use also makes diseases such as age-related macular degeneration an attractive option to begin clinical trials with stem cells. The ranges of clinical and diagnostic tools also help provide the necessary efficacy and safety data to move trials forward [159].

Status of Efforts to Differentiate Stem Cells into Photoreceptors and RPE

As a general principle, patients who could directly benefit from cell-based therapies with retinal degenerative disease such as RP and AMD will require replacement of lost photoreceptor cells, RPE, and possibly choriocapillaris [160]. Korte et al. have shown the choriocapillaris can regenerate if areas of absent RPE can be repopulated with new RPE. Thus the clinical need here is to promote the differentiation of stem cells into photoreceptors (rods and cones) and RPE.
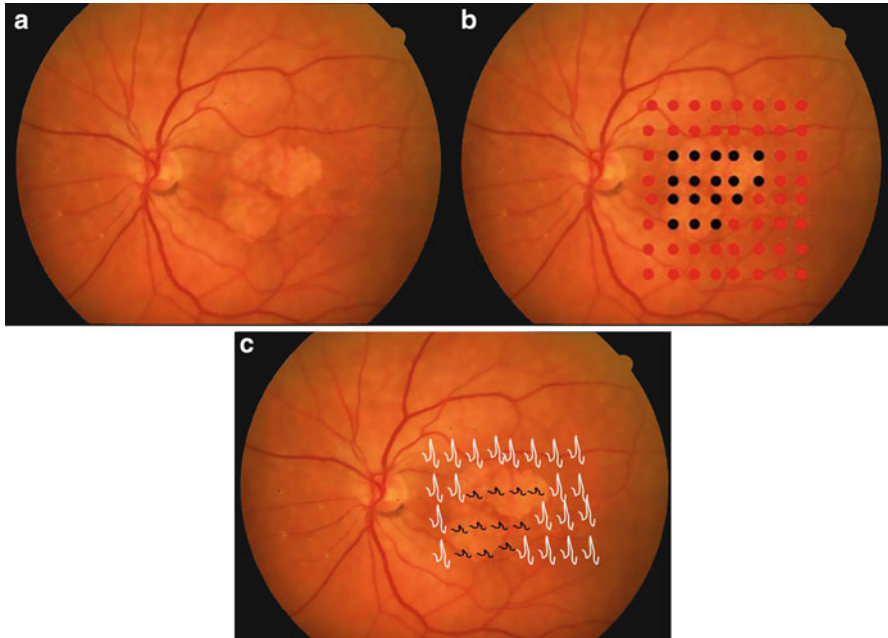
Fig. 1.4 (**a**) Microperimetry can be used to assess macular sensitivity and retinal fixation in normal and atrophic areas of retina by providing a retinal visual function map on a selected, localized fundus location with preset or customized scan patterns. (**b**) In this technique, the retina is illuminated with small spot sizes under direct visualization; this allows the examiner to demonstrate decreased retinal sensitivity in regions of geographic atrophy (*Right panel*, *black circles*) and normal sensitivity in adjacent regions (*Right panel*, *red circles*). (**c**) Multifocal electroretinography (mERG), which allows for topographical measure of electrical activity in distinct areas of the retina, can be used to monitor disease progression and efficacy of therapy. In retinal degenerations, there is typically a decrease in amplitude, or absent ERG signal, in areas of retinal dysfunction (*black tracings*)

In dry AMD, loss of vision arises from loss of RPE and photoreceptors with secondary atrophy of choriocapillaris. A potential treatment for AMD and inherited disease that affect the RPE and photoreceptors would be cell replacement therapy, but one significant hindrance to the clinical use of cell transplantation for treatment of retinal degenerations is the availability of a source of replacement cells. Although RPE derived from prenatal and postnatal tissue has been isolated and induced to grow in vitro, such sources are limited and vary in terms of quality and expansion capacity [141, 161–163]. Moreover, it has been demonstrated that postmitotic photoreceptor precursor cells can be derived from tissue of the early postnatal mouse retina (P1–P5) [164, 165]. However, equivalent retinal cells in humans would have to be derived from second-trimester fetuses. While these studies provide solid evidence that transplantation strategies show great potential, an approach such as this would have ethical implications as well as the problem of a limited reservoir of donor cells [165].
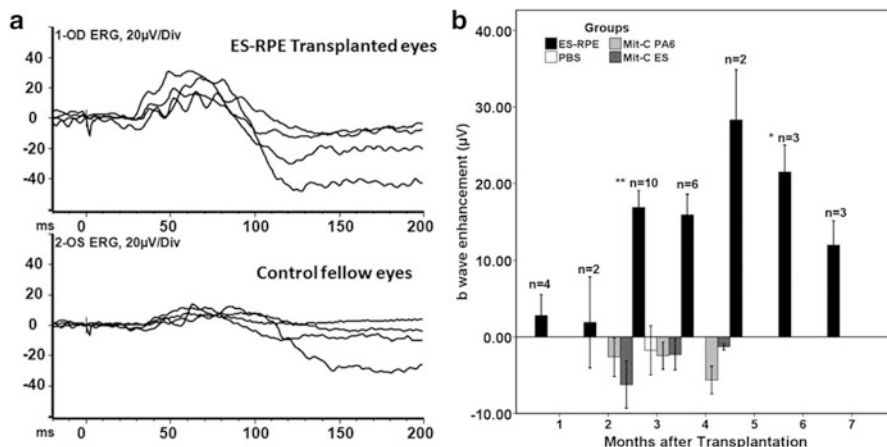
Fig. 1.5 ERGs of $Rpe65^{rd12}/Rpe65^{rd12}$ mice after subretinal transplantation with ES cell-derived retinal pigment epithelial (RPE)-like cells confirm functional rescue. (**a**) ERG from mice after 3 months transplantation. Eyes transplanted with ES cell-derived RPE-like cells (*upper*) showed higher b-wave amplitudes compared with control fellow eyes (*lower*). Traces represent readings from different mice. (**b**) b-wave enhancement in mice 1–7 months post-transplantation, as indicated by *black solid bars*. b-wave enhancement is defined as the difference in maximum ERG responses of transplanted and control fellow eyes (μV). Unpaired t tests were performed for paired differences in b-wave peaks between transplanted and control eyes. At 3 and 6 months post-transplantation, ERGs from transplanted eyes show a statistically significant rescue effect (**$P = 0.001$ and *$P = 0.038$, respectively). Although the difference was not statistically significant at 4, 5, and 7 months after transplantation, the b-wave amplitudes in the transplanted eyes were consistently higher than the control fellow eyes. The number of mice analyzed per time point is indicated. ERGs were performed on both eyes (injected and control) simultaneously. There is no statistically significant difference between injected and control eyes in the other three control groups. *White bar*, b-wave enhancement in PBS injected mice; *light-shaded bar*, b-wave enhancement in mitomycin-C treated PA6 cell transplanted mice; and *dark-shaded bar*, b-wave enhancement in mitomycin-C treated undifferentiated ES cell transplanted mice. *ES-RPE* ES cell-derived RPE-like cells, *Mit-C* mitomycin-C, *ES* embryonic stem, *PBS* phosphate-buffered saline. Reproduced from Wang et al. [158]

Stem cells are an excellent source of cells for replacement therapy given the limited reservoir of donor cells for RPE replacement strategies, lack of regeneration of photoreceptors, and variation in success of autografts. Stem cells have been isolated from a variety of sources including embryo and adult eye [164, 166]. Human embryonic stem cells (hESC) are being investigated as a potential source of photoreceptors and RPE and are promising candidates for therapeutic use.

As mentioned, the strategy in cell replacement therapy using stem cells is to differentiate these cells into photoreceptors or RPE. The extracellular environment plays a critical role in the differentiation of stem cells into the target cell type and extracellular matrix can differentiate cells into the adjacent cell layer. For example, hESCs cultured on a monolayer of cells derived from mouse calverium can be induced to a neural fate and express neural progenitor markers such as paired boxed protein (PAX)-6, neurofilament, and glial fibrillary acidic protein (GFAP) (Fig. 1.6) [167]. Similarly, hESCs cultured on a monolayer of RPE (ARPE19) cells
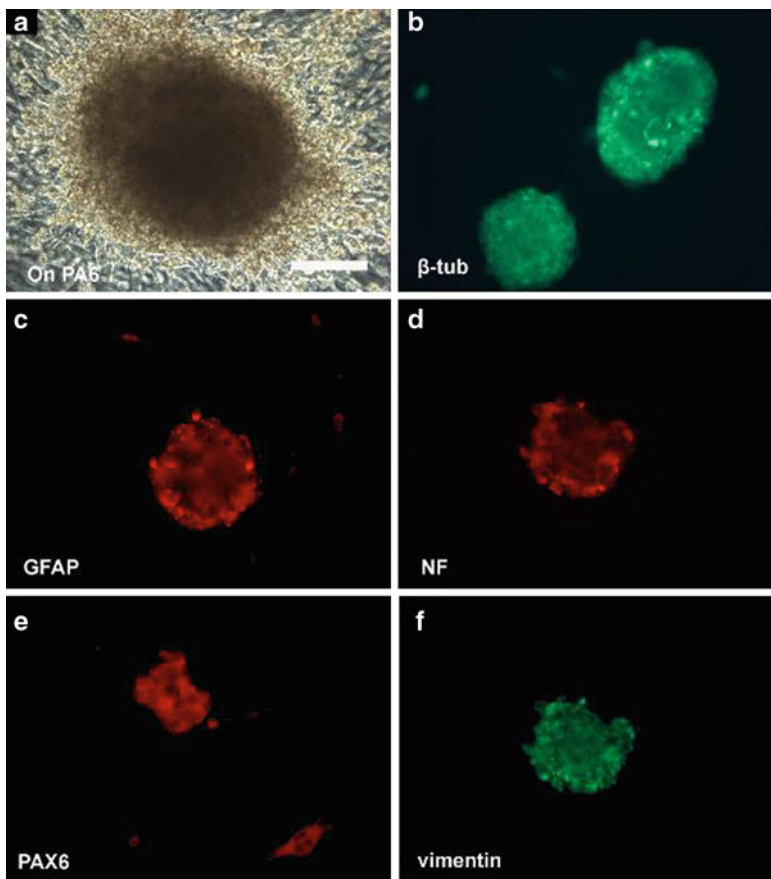
Fig. 1.6 Expression of neural progenitor markers after culturing human embryonic stem cells on mouse PA6. (**a**) Human embryonic stem cells became multilayered and formed pigmented spheres after culturing on mouse PA6 cells for 13 days. Immunofluorescence staining of the spheres demonstrated the presence of several neural progenitor markers including β-tubulin III (>88%) (**b**), GFAP (**c**), neural filament NF200 (>90%) (**d**), PAX6 (>88%) (**e**) and vimentin (**f**). *Bar* = 100 μm

can be induced to express neural retinal markers such as vimentin, neurofilament, and cone–rod homeobox (CRX), which is essential in early photoreceptor development (Fig. 1.7) [167]. Varying extracellular matrix environs such as laminin, matrigel, and/ or vitronectin and fibronectin can also induce embryonic stem cells toward a neural progenitor or RPE fate. Embryonic stem cells cultured on laminin, vitronectin, and fibronectin can be induced to express neural progenitor markers such as neurofilament and neural retina-specific leucine zipper (NRL), an intrinsic regulator of photoreceptor development (Fig. 1.8). These cells can also be induced to express RPE markers such as tight junction protein ZO-1 and bestrophin when cultured on
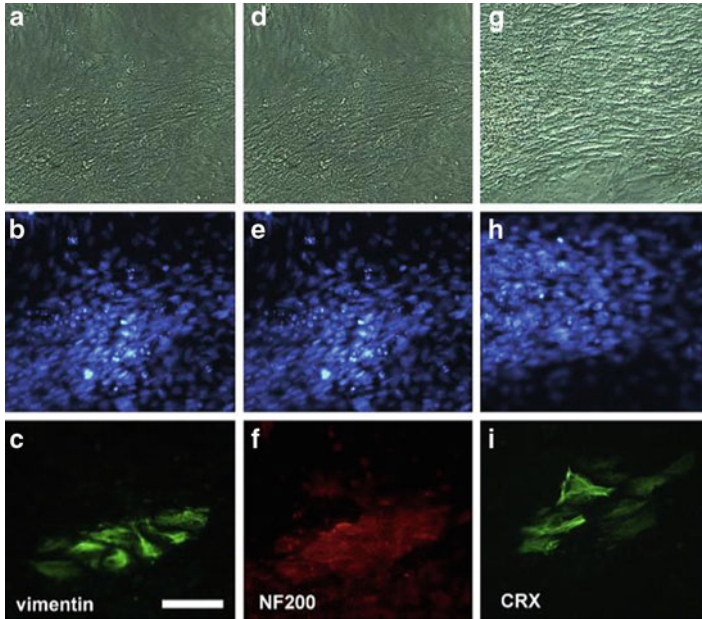
Fig. 1.7 Generation of retinal precursors from neural progenitors after culturing human embryonic stem cells on ARPE19 cells for 10 days. *Top row*, phase-contrast micrographs; *middle row*, nuclei in both ARPE19 and progenitors stained with DAPI. Progenitors expressed neural progenitor marker vimentin (C) and neural filament 200 (F) and photoreceptor-specific protein CRX (I), which is essential during early photoreceptor development. *Bar* = 50 µm

matrigel (Fig. 1.9). Stem cells grown on human Bruch's membrane have also been induced to differentiate to RPE (Fig. 1.10) [167].

Current Status of Human Stem Cells in Clinical Trials

Current clinical trials are underway to evaluate the potential of stem cell therapy in humans. Advanced Cell Technology, Inc. initiated a phase 1 clinical trial in humans in 2011 for the treatment of retinal degenerative disorders [141]. This is the first FDA approved trial for the treatment of macular degeneration using RPE derived from human embryonic stem cells. RPE cells were derived from a single donor human embryonic stem cell line. The preliminary report details the phase 1 trial being conducted to test the safety and tolerability of hESC-RPE in patients with advanced-stage Stargardt's macular dystrophy and dry age-related macular degeneration. Briefly, a human embryonic stem cell (MA09) line was first used to generate hESC-derived RPE, which were then characterized and tested for pathogen contamination. After pars plana vitrectomy, submacular injections of 50,000 cells were used to treat the patient. Patients were immune suppressed with low-dose
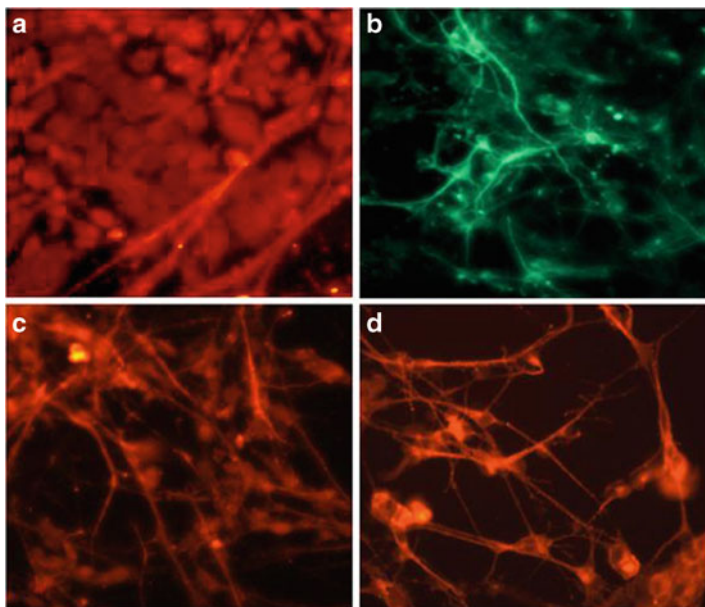
Fig. 1.8 Expression of neural progenitor markers after culturing mouse embryonic stem cells on poly-D-lysine, laminin, vitronectin, and fibronectin. Cells expressed photoreceptor marker NRL (**a**) and neural progenitor markers β-tubulin (**b**) neurofilament 200 (**c**) and vimentin (**d**)
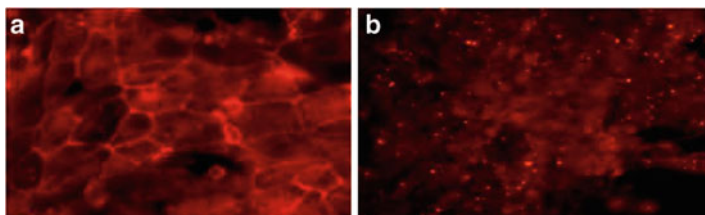
Fig. 1.9 Expression of retinal pigment epithelium (RPE) markers after culturing mouse embryonic stem cells on matrigel. Cells expressed RPE makers Z0-1 (**a**) and Bestrophin (**b**)

tacrolimus and mycophenolate mofetil 1 week before surgery and continued for 6 weeks postsurgery. After 6 weeks patients discontinued tacromilus and continued a mycophenolate regimen for 6 more weeks. Both patients tolerated the injection without signs of postoperative inflammation, rejection, or tumorigenicity at time of the report (4 months follow-up). In the patient with Stargardt's macular dystrophy, transplanted cells attached to Bruch's membrane and persisted throughout the observation period; there was possible visual improvement in the injected eye as shown by visual acuity and Goldmann visual field test. In the AMD patient no clinically detectable sign of successful transplantation was observed, although the patient did not comply with the immunosuppressive drug regimen [168]. Interestingly, there was mild visual improvement in both eyes of the patient with AMD as
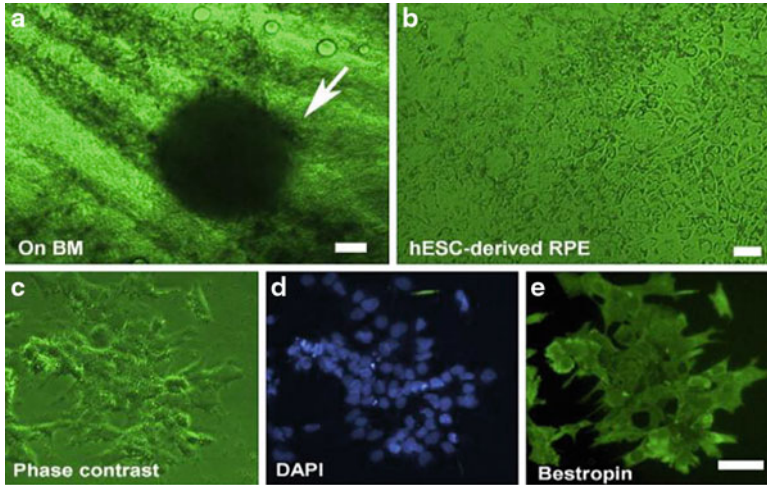
Fig. 1.10 Induction of RPE markers in human embryonic stem cells cultured on human Bruch's membrane. (**a**) Cluster of pigmented human embryonic stem cells 4 days after growing on human Bruch's membrane explants (*arrow*). (**b**) Phase-contrast micrograph of flattened pigmented epithelium-like cells on human Bruch's membrane. (**c**)–(**e**) hESC-derived RPE under phase contrast, DAPI and Bestrophin staining, respectively. *Bar = 50 μm* in (**a**), (**c**)–(**e**); *bar = 20 μm* in (**b**)

shown by visual acuity and Goldmann visual field test. It is unclear whether these visual improvements are due directly to the cell transplant or a secondary cause such as the immunosuppressive drugs or a placebo effect [141]. Nevertheless, these important studies demonstrate that stem cells can be transplanted into the subretinal space in humans without abnormal proliferation, teratoma formation, graft rejection, or other untoward pathological reaction or safety signal.

These short-term results in only two patients, are preliminary but provide valuable proof-of-concept evidence for future treatment of macular degeneration and Stargardt's disease in humans. Long-term follow-up in a larger cohort of patients is needed to draw more meaningful conclusions from this trial [168]. Further trials are also needed to determine the optimal number of transplanted cells, immunosuppression regimens, and disease stage for transplantation [168].

Induced Pluripotent Stem Cells as a Therapeutic Option

The recent development of induced pluripotent stem (iPS) cells holds great promise as a potential reservoir of cells for the treatment of age-related macular degeneration and other disorders. Induced pluripotent stem cells were initially generated in 2006 by the Yamanaka group and the technology has had dramatic implications both from an ethical and scientific standpoint [169]. The technology is a significant advancement over prior technology, as it allows researchers to generate pluripotent cells for
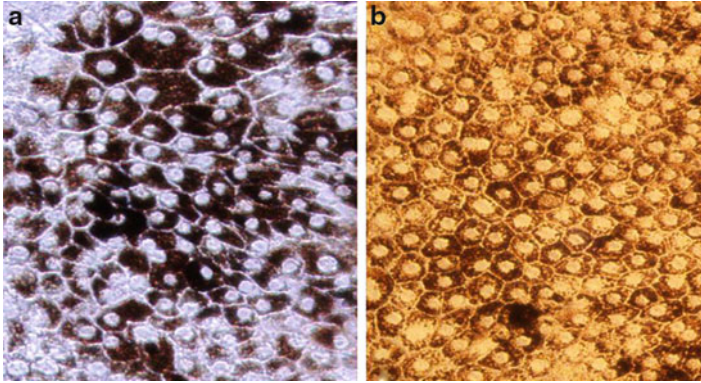
Fig. 1.11 Generation of RPE-like cells from human-induced pluripotent stem (iPS) cells. RPE derived from iPS grown on PA6 feeder cells (**a**, **b**). PA6 feeder cells exhibit stromal-derived inducing activity (SDIA), which promotes differentiation into RPE (Image courtesy of Stephen Tsang)

potential therapeutic use without the controversial use of embryos. These cells are generated by reprogramming adult somatic cells using transcriptional regulators such as *SOX2*, *OCT3/4*, and *Klf4* [169, 170]. These cells are then reprogrammed with similar potential as embryonic stem cells and are capable of differentiating into three germ layer cell types (mesoderm, ectoderm, and endoderm) [169, 171, 172]. These iPS cells hold great promise for the generation of RPE and photoreceptors for cell replacement therapy and create a new paradigm as a novel reservoir. Tsang et al. have generated RPE-like cells from human iPS (Fig. 1.11). Several other groups have used iPS technology to generate photoreceptors and then transplant these cells into animal models of retinal degeneration [173–175].

Induced pluripotent stem cells can also provide a platform to study disease through the use of patient-specific iPS cells. Through the generation of iPS cells from patients with specific diseases, models can be developed to express particular disease phenotypes which can then be used to understand pathophysiology of disease and determine the efficacy of therapeutic interventions [176]. These models can also be developed to help understand human inherited diseases given their clinical and genetic heterogeneity [177]. Cells derived from a particular patient can be used as a biological tool for drug discovery and toxicity testing of therapeutic agents, providing a new paradigm for personalized medicine [176].

Utilizing iPS cells as a tool for cell replacement therapy could also reduce the possibility of immune rejection given their autologous nature. Use of patient-specific iPS-derived RPE, generated from somatic cells of the potential transplant recipient with geographic atrophy, has one major and important theoretic advantage over other potential cell sources, namely, the avoidance of graft rejection. This is an important advantage, since long-term systemic immune suppression is poorly tolerated in elderly patients. Although the presence of anterior chamber-associated immune deviation (ACAID) confers some immune privilege in the subretinal space,

allogeneic RPE will undergo graft rejection after subretinal transplantation unless immune suppression is used [178, 179]. Use of patient-specific iPS may circumvent graft rejection, which is one of the major challenges to ensuring graft survival in the subretinal space.

Challenges remain to the successful use of iPS cells. Induced pluripotent cells derived from the affected patient contain the predisposing mutation that caused the disease. This can provide a unique disease model but the mutation may also impede the function of the transplanted cells. These stem cells may have to first be repaired by targeted gene therapy or other techniques prior to transplantation. Additional work is also needed to translate the advances of iPS cells into clinical trials to assess safety and efficacy. Better understanding of iPS cell technology and refining the methodology of their generation will have a significant impact on retinal degenerations and regenerative medicine.

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