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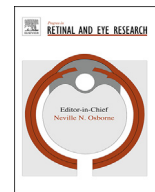Zhu, Danhong
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Stem cell based therapies for age-related macular degeneration: The promises and the challenges

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ABSTRACT

Age-related macular degeneration (AMD) is the leading cause of blindness among the elderly in developed countries. AMD is classified as either neovascular (NV-AMD) or non-neovascular (NNV-AMD). Cumulative damage to the retinal pigment epithelium, Bruch's membrane, and choriocapillaris leads to dysfunction and loss of RPE cells. This causes degeneration of the overlying photoreceptors and consequential vision loss in advanced NNV-AMD (Geographic Atrophy). In NV-AMD, abnormal growth of capillaries under the retina and RPE, which leads to hemorrhage and fluid leakage, is the main cause of photoreceptor damage. Although a number of drugs (e.g., anti-VEGF) are in use for NV-AMD, there is currently no treatment for advanced NNV-AMD. However, replacing dead or dysfunctional RPE with healthy RPE has been shown to rescue dying photoreceptors and improve vision in animal models of retinal degeneration and possibly in AMD patients. Differentiation of RPE from human embryonic stem cells (hESC-RPE) and from induced pluripotent stem cells (iPSC-RPE) has created a potentially unlimited source for replacing dead or dying RPE. Such cells have been shown to incorporate into the degenerating retina and result in anatomic and functional improvement. However, major ethical, regulatory, safety, and technical challenges have yet to be overcome before stem cell-based therapies can be used in standard treatments. This review outlines the current knowledge surrounding the application of hESC-

RPE and iPSC-RPE in AMD. Following an introduction on the pathogenesis and available treatments of AMD, methods to generate stem cell-derived RPE, immune reaction against such cells, and approaches to deliver desired cells into the eye will be explored along with broader issues of efficacy and safety. Lastly, strategies to improve these stem cell-based treatments will be discussed.

1. Introduction

Age-related macular degeneration (AMD) is the leading cause of vision loss and inability to read, drive, and perform daily tasks among people 50 years of age and older around the world. In the United States, AMD affects nearly 2 million Americans (Kahn et al., 1977; Ambati et al., 2003; Bressler, 2004; Friedman et al., 2004; Herm, 2008). This number is expected to grow to 3 million by the year 2020 as the number of elderly individuals increases in the U.S. (Friedman et al., 2004). It is estimated that about one third of individuals aged 75 and older suffer from some form of AMD (Zarbin, 2004). While the disease is particularly prevalent in developed countries, especially in individuals with Caucasian ethnicities, it is becoming a global health concern (Ambati et al., 2003) due to increases in life expectancies worldwide, with the number of people becoming blind or losing substantial eyesight because of AMD predicted to increase steeply (Araujo Filho et al., 2008). Additionally, more attention is now being focused on AMD as expectations for a high quality of life, including functions such as driving and reading, increase in communities around the world.

Early AMD is characterized by the thickening of Bruch's Membrane and deposits under the retinal pigment epithelium layer and by pigmentation changes in the macula. More advanced stages of the disease demonstrate either subretinal neovascularization or atrophy of the retina and RPE. Based on the absence or presence of blood vessel growth progressing from the choroidal side towards the retina, the disease is broadly subdivided into non-neovascular and neovascular AMD. Non-neovascular AMD (NNV-AMD), also known as dry AMD, accounts for the majority of AMD cases (approximately 80%). NNV-AMD is clinically identifiable by the presence of basal laminar deposits and drusen under the RPE layer, as well as by changes in RPE pigmentation, as mentioned above. The presence of drusen, in long term, leads to attenuation of the overlying RPE and photoreceptors. In advanced stages of the disease, the drusen may disappear and well demarcated areas of RPE and photoreceptor loss called geographic atrophy (GA) appear in their place (Fig. 1) (Ambati et al., 2003; Ouyang et al., 2013). On the other hand, a subset of patients with drusen progress to the neovascular form of AMD (NV-AMD) (also known as exudative or wet AMD) where defects in the underlying Bruch's membrane (BM) provide access for abnormal capillaries to grow into sub-RPE and subretinal areas. Vision loss in patients with NV-AMD can be sudden and severe and occurs mainly because of fluid leakage or bleeding from highly-permeable subretinal or sub-RPE vascular networks. This less common, but more visually devastating subtype of AMD, accounts for about 20% of patients with AMD (Kahn et al., 1977), but 90% of AMD patients with severe visual loss.

Despite the increasing burden of AMD on the global health economies (Day et al., 2011), there are limited prevention options and no treatments available for patients with NNV-AMD, although effective, but laborious, treatments exist for patients with NV-AMD. Strong evidence shows that lifestyle and dietary modification can slow the progression of early AMD to advanced forms (Clemons et al., 2005; Age-Related Eye Disease Study Research et al., 2007). On the other hand, for NV-AMD, effective treatments are available using anti-vascular endothelial growth factor (anti-VEGF) (Gragoudas et al., 2004) agents, although this typically requires tedious monthly injections. There is also evidence that the underlying atrophic component of AMD still progresses despite using anti-VEGF therapy (Young et al., 2014). So, while anti-VEGF agents have revolutionized the clinical management of NV-AMD diagnosed early in their disease course, many patients with advanced NV-AMD and almost all patients with advanced NNV-AMD continue to lose vision because no treatment option is currently available for them.

Macular translocation surgery (Machemer and Steinhorst, 1993; de Juan et al., 1998; Fujikado et al., 1998; Pieramici et al., 2000) and RPE transplantation surgery (Algvere et al., 1994; Stanga et al., 2002; van Meurs and Van Den Biesen, 2003) have provided compelling evidence that healthy RPE can support photoreceptor survival and visual function in human patients with choroidal neovascularization (CNV) due to AMD and high myopia (Binder et al., 2007). While technical challenges, including proliferative vitreoretinopathy and torsion, have limited the practicality of these surgeries, the attempts were promising, as demonstrated by decreased visual loss rates and even improvement of vision in some patients (Algvere et al., 1997; Stanga et al., 2001; Fujii et al., 2002). This provided proof-of-principle that replacing dead or dying RPE with healthy RPE can rescue vision in patients with advanced AMD. Limited autologous sources of maculae and healthy RPE sheets for transplantation, and the fact that autologous sheets of peripheral RPE would share genetic risks identical to those of the patient's posterior pole RPE cells, have prevented wide acceptance of autologous transplantation surgeries. The search for other sources of healthy RPE has extended to harvesting RPE from fetal or adult donor eye tissue; however, fetal and adult RPE sources are also limited, and harvesting them confers significant technical and ethical challenges.

The study of differentiation of functional RPE and retinal progenitor cells from human pluripotent stem cells in the early 2000s opened a promising approach to generating a virtually unlimited supply of RPE and photoreceptor cells for replacement therapies (Lund et al., 2006). During the last decade, the stem cell-based cellular therapeutics for retinal degenerative diseases have been mainly focused on (1) replacing RPE to maintain the supportive function of the RPE layer, (2) replacement with retinal progenitor cells (RPCs) to regenerate lost retinal elements, including photoreceptors, or (3) combining RPE and RPC replacement in advanced stages where both RPE and retinal elements are lost. Significant research efforts have focused on finding the ideal methods for efficiently deriving RPE and RPC from embryonic and induced pluripotential stem cells, transplanting these cells into the subretinal space, and preventing immune rejection of the transplanted cells. Multiple excellent reviews have discussed hESC-RPE and iPSC-RPE derivation in depth (Blenkinsop et al., 2012; Carr et al., 2013; John et al., 2013; Melville et al., 2013; Ramsden et al., 2013). The aim of this review is to present an up-to-date summary of hESC-RPE and iPSC-RPE derivation with a focused discussion on the technical and biologic challenges of stem cell-derived RPE and RPC transplantation for the treatment of AMD. We begin with a limited review of RPE cell function and the pathogenesis of AMD and its clinical subtypes. We will not discuss the pathogenesis
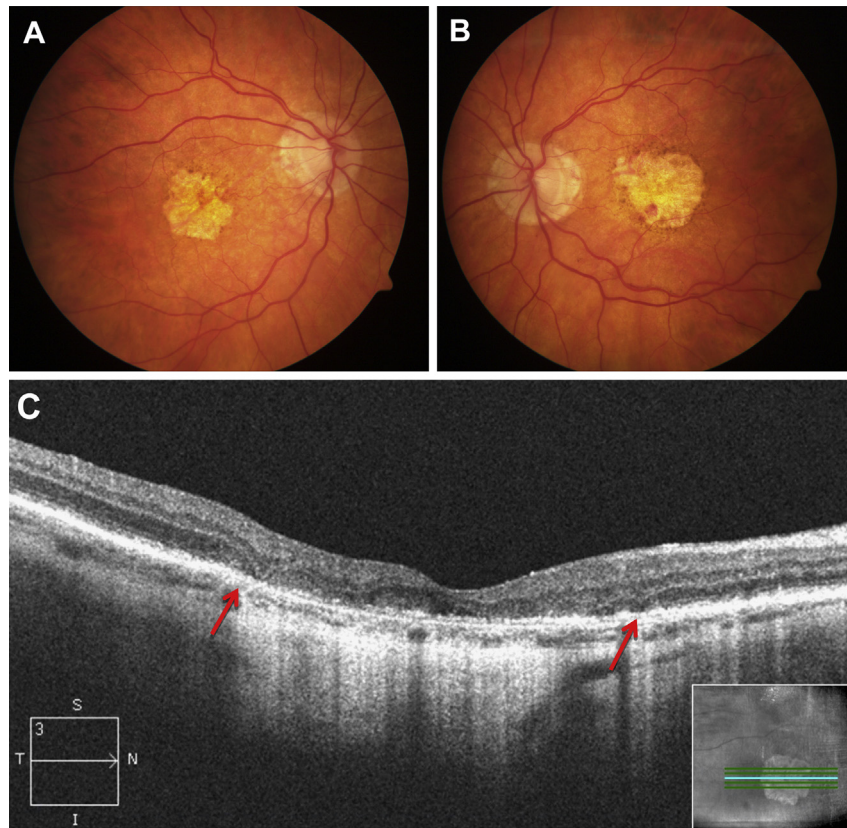
Fig. 1. Geographic atrophy (GA) in a patient with non-neovascular age-related macular degeneration (NNV-AMD), also known as dry AMD. (A and B) Fundus photographs of a patient with NNV-AMD showing sharply demarcated boundaries of GA. (C) Optical coherence tomography (OCT) reveals loss of the retinal pigment epithelium (RPE) layer (between the red arrows). The outer retinal layers are also apparently atrophic in the GA area, resulting in retinal thinning.

of the disease in detail as other reviews have previously covered this topic (Zarbin, 2004; Ding et al., 2009). Next, we will explore methods for stem cell-RPE derivation and delivery, including addressing immunologic questions, and evaluate stem cell-derived RPE in terms of safety and efficacy. Finally, current and prospective clinical trials, future challenges, and progress in bringing stem cell therapies to the bedside will be covered.

2. RPE cells and their role in vision

The RPE is a single layer of cells that forms the outer blood-ocular barrier located between the retinal photoreceptors and the choriocapillaris. RPE cells form a monolayer of hexagonally-shaped pigmented cells overlying a basement membrane that is, in turn, part of a pentalayer extracellular matrix called Bruch's Membrane. Bruch's Membrane is comprised of the basement membrane of the RPE, the choriocapillaris, and the loose and dense elastic connective tissues in between. RPE cells are a highly polarized cell type. The basal side of the cells attaches to the basement membrane with hemidesmosomes while the apical side, containing abundant microvilli, faces and surrounds the photoreceptor outer segment tips. It is estimated that each RPE cell faces 20–55 photoreceptors in the macular region (Binder et al., 2007). The space between the RPE microvilli and the rod and cone outer segments is filled with an interphotoreceptor matrix that, together with the microvilli, holds the retina in close apposition to the RPE (Hollyfield, 1999). The lateral sides of the RPE cells are tightly joined to each other by tight junctions, impermeable to the transport of water, electrolytes, and larger molecules under normal conditions (Campbell and Humphries, 2012).

RPE cell density and size vary by the cell's location in the eye.

Macular RPE cells are smaller in area, more columnar (taller), and more pigmented than peripheral RPE cells (Panda-Jonas et al., 1996). Despite annual loss of about 0.3% of the macular RPE cells by age, the macular RPE cell density actually increases with age, probably due to the migration of peripheral cells toward the center (Binder et al., 2007).

The RPE is critical for normal photoreceptor (PR) function. RPE cells provide nutrients and oxygen to PRs, phagocytize rod and cone outer segments, and are central to the regeneration of photopigment. The RPE is a highly pigmented layer that absorbs stray light within the eye while helping to dissipate the heat in the retina generated by this light and the phototransduction process. Moreover, RPE cells secrete cytokines and growth factors in a polarized fashion that is critical for maintenance of the choriocapillaris and retina. Specifically, VEGF produced by the basal side of the RPE cells is vital to the health of the choriocapillaris. Additionally, pigment epithelium-derived factor (PEDF) and transforming growth factor-beta (TGF-β), secreted mainly by the apical side of RPE cells, perform a variety of functions, including providing an immuno-suppressive microenvironment in the subretinal space (Zamiri et al., 2006; Zhu et al., 2011). Thus, all this supports the hypothesis that it is primarily dysfunction of the RPE that initiates photoreceptor atrophy and choriocapillaris loss in the course of AMD due to the critical role of RPE cells in maintaining normal retinal and choroidal physiology.

3. Age-related macular degeneration: pathogenesis and clinical types

Because an in-depth discussion of the pathogenesis of AMD is beyond the scope of this chapter, only a summary of the most

relevant findings will be discussed here. AMD is a multifactorial disease where both environmental and genetic causes play a role in its development. Although the primary site of pathologic insult in AMD is not clear, histologic, biochemical, and genetic analysis support involvement of oxidative damage, inflammatory changes (at the levels of the choriocapillaris, BM, and RPE layer), and the gradual accumulation of indigestible material within the RPE cells (Swaroop et al., 2009; Zarbin and Rosenfeld, 2010). All of these factors culminate in progressive atrophy of RPE and the choriocapillaris–choroid complex, eventually leading to photoreceptor loss (Binder et al., 2007).

RPE is under consistent oxidative stress (Zarbin and Rosenfeld, 2010). Post-mortem eyes from patients with GA show accumulation of oxidative damage biomarkers, including lipid peroxidation products and DNA breakdown, suggesting possible defects in the oxidative defense system (Shen et al., 2007). Other evidence supporting a role of oxidative damage in the pathogenesis of AMD comes from epidemiologic studies showing that high dietary antioxidant consumption decreases the chance of developing advanced AMD. In addition, smoking, a major oxidative stress factor, has been demonstrated to be a major risk factor for developing AMD in multiple studies (Myers et al., 2014). Accumulation of lipofuscin from non-degradable components of cone and rod outer segments seems to play a major role in RPE damage and death. Activated complement components are detected in drusen and in Bruch's membrane. In addition, the presence of inflammatory cells in Bruch's membrane in patients with AMD indicates that chronic inflammation plays a role in AMD pathogenesis too. Multiple mutations in complement system regulatory components are associated with the increased risk for drusen, CNV, and GA formation (Hageman et al., 2001; Chakravarthy et al., 2013). On the other hand, choroidal blood flow is consistently compromised in patients with AMD and has a direct correlation with the severity of the disease (Grunwald et al., 2005). Although it is suggested that reduced choroidal perfusion starts before the development and progression of AMD, it is also possible that subclinical RPE dysfunction results in decreased VEGF production and subsequent choriocapillaris atrophy.

Local immune dysregulation at the level of the RPE and BM, and constitutional vulnerability to aging stress, are known underlying events leading to damage in AMD (Ding et al., 2009). About one-half of the heritable forms of AMD is associated with defects in the complement system, namely, regulatory component, complement factor H (Edwards et al., 2005; Haines et al., 2005; Klein et al., 2005). Abnormal complement factor H variant 402H does not regulate complement activation via the alternative pathway as efficiently as the main allele does. This results in a greater degree of inflammatory damage to the retina and RPE following retinal injury (Hageman et al., 2001). In addition, other members of the regulatory complement protein group including components C3, C2, factor B, and factor I are implicated in the pathogenesis of AMD (Ambati et al., 2013). A major inflammatory pathway in the pathogenesis of GA is found to be RNase III DICER1 deficiency that leads to the accumulation of cytotoxic Alu RNA in RPE cells and up regulation of LRP3 inflammasome causing RPE cell death via interleukin-18 (IL-18)-mediated MyD88 signaling and caspase-8 activation (Dridi et al., 2012; Kerur et al., 2013; Kim et al., 2014; Tarallo et al., 2012). Recent advances in haplotype mapping and single nucleotide polymorphism (SNP) analysis of complement H and C factors have broadened our view of how genetic predisposition to local inflammatory dysregulation compromises the integrity of the BM-RPE complex, culminating in loss of RPE, overproduction of VEGF, and development of subretinal neovascular membranes. In addition, mutations in Age Related Macular Degeneration Susceptibility 2 (ARMS2)/High Temperature Required Factor A1 (HTRA1) genes, complement component 2/complement factor B (C2/BF, 6p21) genes, and complement component 3 (C3, 19p13) genes are believed to contribute to the pathogenesis of AMD (Ding et al., 2009; Richardson et al., 2009). Also, the role of genes involved in the high density lipoprotein cholesterol pathway (such as Lipase C (LIPC), cholesterol ester transfer protein (CETP), and ATP-binding cassette sub-family A member 1 (ABCA1)), extracellular matrix biology (such as TIMP3, near collagen type X alpha 1 precursor/fyn related kinase (COL10A1/FRK) and collagen type XIII alpha 1 (COL8A1), and angiogenesis pathway (such as VEGF-A and TGF-βR 1) and other significant loci (such as TNFRSF10A, APOE, IER3/DDR1, SLC16A8, RAD51B, ADAMTS9/MIR548A2, and B3GALTL) in the pathogenesis of AMD have been supported in genome-wide association studies (GWAS) and other reports recently reviewed by Sobrin and Seddon (2014).

Although AMD is traditionally typified as "dry" and "wet", emerging evidence shows that both types involve a common pathophysiologic background and share similar initiating molecular and cellular alterations. Insight into the possible genetic drivers of AMD suggests that genetic risks of NV-AMD and NNV-AMD are also similar (Fritsche et al., 2013). Despite this common genetic and clinical background, the differentiation of dry AMD and wet AMD is clinically relevant because of their distinct clinical presentations, natural histories, and therapeutic options. The AMD classification presented below is based on the clinical appearance at the macula.

3.1. Early and intermediate AMD: drusen

Age-related changes in RPE and photoreceptors include diminished photoreceptor density, reduced size and number of intra-RPE melanin granules, and lipofuscin granule formation (Zarbin, 2004). Deposition of small amounts of amorphous material between the RPE plasma membrane and basal lamina, called basal laminar deposits, and between the basal lamina of RPE and the inner collagenous layer of Bruch's membrane, called basal linear deposits as well as choriocapillaris atrophy are frequently seen as part of normal aging and are not pathognomonic for AMD (Zarbin, 2004).

Early AMD is characterized by the thickening of Bruch's membrane and the appearance of sub-RPE deposits called drusen. Bruch's membrane thickening and lipid and protein build-up are believed to interfere with free fluid efflux from RPE to choriocapillaris and impose stress on the RPE. Discrete build-ups of the deposits are individually known as druse and can vary in size and texture. Although a hallmark of AMD, drusen are found in the normal aging eye, as stated before, and their role in the pathogenesis of AMD is not fully understood. These drusen are hard to identify in the beginning; they appear as semitransparent small dots under the retina and build up over time. They are clinically identified as sub-RPE yellow-white deposits that, based on the characteristics of their size, edges, and the risk imposed for advanced disease, are typically divided into two main morphologic phenotypes: hard and soft. Small drusen are ≤63 μm in diameter; this amounts to half of the width of a retinal vein when it passes over the optic nerve head margin. Numerous small drusen are considered a moderate risk factor for AMD (Macular Photocoagulation Study Group, 1997). Small drusen may regress and leave a region of localized RPE atrophy in place or progress to larger drusen. Intermediate-sized (64–124 μm in diameter) and large (>125 μm) drusen build up on smaller drusen and are generally soft, meaning that they have indistinct margins and tend to become confluent over time. Hard drusen are rich in apolipoprotein content and much less fragile upon dissection compared to soft drusen (Rudolf et al., 2008). The presence of hard drusen in the macula appear to increase the incidence of soft drusen and pigmentary changes in the RPE, that in turn, are more likely to

progress to the advanced stages of AMD.

The presence of multiple intermediate drusen or one or more large confluent drusen is considered to be a major risk factor for the progression of RPE/BM disturbances and AMD (Zarbin, 2004). Drusen may become calcified, regress, or coalesce to form a large drusenoid pigment epithelial detachment that is an ominous sign of AMD progression (Klein et al., 2008). The presence of intermediate and large drusen, soft drusen, and bilateral drusen increases 5-year and 10-year incidence of progression to advanced AMD. In the presence of bilateral drusen and good vision in both eyes, the annual incidence of new atrophic or exudative lesions is approximately 8% over the next 3 years (Holz et al., 1994). As disease progresses to intermediate phases, medium-sized drusen are seen with more prominent pigmentary changes. Intermediate AMD can progress to severe AMD and advanced stages of disease. Here, GA is characterized by patchy areas of RPE loss with overlying photoreceptor attenuation and/or exudative AMD with abnormal neovascular membrane growth accompanied by hemorrhage and exudation.

A clear understanding of the progression of early AMD to advanced forms is highly relevant for selecting the optimal time to perform a stem cell-derived RPE transplantation, as the ideal patient for such replacement therapy may be one with a high probability of progression to advanced AMD yet still has a fairly intact photoreceptor layer. Thus, predicting the probability of progression based on clinical and imaging features of drusen is an area of active research that should have a major impact on the field of RPE replacement therapy.

3.2. Neovascular AMD

With cumulative degenerative changes of early and intermediate AMD in choriocapillaris, Bruch's membrane, and RPE, some patients progress to the more advanced forms of the disease in the form of GA and NV-AMD. NV-AMD, also known as exudative or wet AMD, is characterized by the growth of new abnormal blood vessels that originate in the choriocapillaris (underlying Bruch's membrane and RPE) and may pass upwards, through Bruch's membrane, to ultimately grow directly under the RPE or retina. Given the origin of these abnormal new vessels, they are also called choroidal neovascularization (CNV). The absence of a competent blood-ocular barrier in CNV lesions leads to plasma leakage and oozing of blood, which is seen as subretinal and intraretinal hemorrhage and edema.

With the earliest signs of the progression of NNV-AMD to NV-AMD, patients generally complain about a rapid and dramatic drop in central vision or the development of new visual distortions. In a fundus examination, new vessels classically appear as a greenish gray discoloration underneath the retina with accompanying intraretinal and subretinal fluid accumulation and hemorrhage. The anatomic position of new vessels in the subretinal or sub-RPE spaces classifies CNVs into two major subtypes - classic and occult - with each having distinct fluorescein angiographic features, clinical presentations, and responses to treatment. Imaging studies, optical coherence tomography (OCT) in particular, have changed the diagnosis and management of AMD during the last decade. Spectral domain-OCT and OCT improvements, such as swept-source OCT coupled with analysis algorithms (e.g., retinal thickness maps and RPE maps), are now essential tools used in the management of NV-AMD.

The natural history and prognosis of NV-AMD is known from the laser treatment era, when the Macular Photocoagulation Study showed 62%–65% of eyes with CNV suffer from CNV progression over a 5 year follow-up period (Virgili and Bini, 2007). While very limited therapeutic options were available about a decade ago and NV-AMD was considered a universally blinding disease, recognition of VEGF as a unique driver for the growth of new subretinal vessels and the consequential advent of anti-VEGF medications has led to a dramatic improvement in NV-AMD treatment. Thus, NV-AMD is now considered a treatable condition, if diagnosed early enough. However, for a subset of patients who suffer from inadequate and late treatment, NV-AMD leads to the loss of photoreceptors and thus permanent loss of vision. When sufficient numbers of photoreceptors remain though, a phase one clinical trial is being planned by Pfizer and University College London to test the safety and efficacy of sheets of hESC-RPE for the treatment of acute loss of vision in NV-AMD. RIKEN in Japan has also started a clinical trial to use iPSC-RPE sheets in patients with NV-AMD.

3.3. Geographic atrophy

Geographic atrophy (GA) refers to patchy areas of RPE loss accompanied by overlying outer retinal atrophy (Ambati et al., 2003; Sunness, 1999). GA may develop after the regression of drusen, attenuation of RPE, flattening of RPE detachment, or regression of CNV. The presence of drusen larger than 250 μm is a major risk factor for the development of GA (Sunness, 1999). Early in its course, GA usually affects the parafoveal retina and is bilateral with a fairly symmetric pattern of RPE loss. GA is responsible for 20% of visual loss in patients with AMD (Ferris et al., 1984). Development of GA is associated with a mean decrease of 7 letters from the baseline VA level compared with 1 letter among matched, early AMD eyes without GA (Brader et al., 2013). GA prevalence increases with age and individuals 75 years of age or older are 14 times more likely to experience geographic atrophy compared to those 60–64 years of age (Buch et al., 2005). Also, GA tends to develop at a greater age than NV-AMD (Buch et al., 2005). In autopsies of patients with AMD, one third show atrophic patches of RPE and overlying photoreceptors (Green and Enger, 1993).

OCT imaging clearly delineates the boundaries of GA by showing thinning of the outer nuclear layer, decreased RPE reflectance, and increased signal intensity in the underlying choroid and choriocapillaris. The choriocapillaris and outer choroidal layers also become atrophic, as shown by OCT studies (Sohrab et al., 2012). Whether choriocapillaris atrophy leads to a diminished oxygen and nutrient supply and ultimate RPE atrophy or vice-versa is an active debate. It is likely that both are involved in the pathogenesis of GA. Fundus autofluorescence (FAF) is a relevant adjunct to color fundus and OCT imaging because it seems to provide specific information regarding the RPE. FAF correlates very well with OCT-based size measurements of GA (Holz et al., 2015). FAF depends on the inherent fluorescent properties of bisretinoids that are produced in photoreceptor cells and accumulate in lipofuscin in the RPE. While the exact cause of autofluorescence changes in AMD are still being debated, strong evidence supports the notion that FAF images depict the extent of RPE atrophy better than color photographs and may provide additional information regarding the areas most at risk of future atrophy.

Accurate prediction of GA progression is important for selecting the optimal time for performing RPE replacement therapy on patients with GA areas abutting the fovea. The mean enlargement rate of GA is reported to be 2.6 mm²/year, with larger areas growing at faster rates (Sunness et al., 2007). It is easier to detect and follow the GA area in autofluorescence scans compared to color photography. While early NNV-AMD shows various patterns of hyperautofluorescence and hypoautofluorescence based on the size, composition, and topography of drusen and drusenoid deposits (Bindewald et al., 2005), GA is characterized by sharply demarcated patches of hypoautofluorescence in affected areas. A more dramatic but less understood finding is the presence of the

hyperautofluorescence halo surrounding GA patches (Holz et al., 1999). Microperimetry studies have correlated these areas of hyperautofluorescent halos with abnormal photoreceptor function, and consequently proposed a pathophysiologic association between these abnormalities and the progression of GA (Schmitz-Valckenberg et al., 2004; Schmitz-Valckenberg et al., 2006). Patterns of hyperautofluorescence surrounding GA patches as studied by Holz et al. showed that GA lesions that are surrounded by diffuse and banded hyperautofluorescence halo tend to have a higher progression rate (Holz et al., 2007). The same group has suggested that, in general, the presence of surrounding, autofluorescent abnormalities has the highest correlation with atrophy progression compared to any other risk factors (including size of baseline atrophy, history of smoking, hypertension, diabetes, age > 80 years, hyperlipidemia, and family history) (Schmitz-Valckenberg et al., 2009). If proven safe and effective in clinical trials, patients with GA lesions approaching the fovea, or those with high risk characteristics of diffuse trickening and banded hyperautofluorescence patterns, may opt for earlier stem cell derived-RPE implantation.

In addition, microperimetry can be used to assess retinal function and its changes over time (Scholl et al., 2004; Wu et al., 2014). Studies from multiple groups have demonstrated decreased retinal sensitivity in subjects with early AMD lesions (Wu et al., 2014) as well as at the retina around GA where autofluorescence changes are seen (Schmitz-Valckenberg et al., 2004). Thus, microperimetry may be helpful for identifying AMD patients who are more vulnerable to losing central vision and are consequently in greater need for earlier replacement therapy. Similarly, changes observed via microperimetry may also be among the first signs showing that a patient has improved retinal function following therapy designed to treat AMD-related vision loss.

4. Current and experimental treatments for AMD

Due to the complex multifactorial nature of the disease, therapeutic options for AMD have evolved slowly. Lifestyle modifications, including smoking cessation and using high-dose antioxidant vitamin and mineral supplements are shown to decrease AMD progression rate. For NV-AMD, repeated injections of anti-angiogenic agents are used.

4.1. Neovascular AMD

For years (and still in use in selected cases), clinicians treated NV-AMD using photocoagulation ablation of extrafoveal new vessels. This method was associated with a high rate of recurrence and damage to the retina with resulting blind spots (Macular Photocoagulation Study Group, 1991). Photodynamic therapy (PDT) with systemic administration of a photosensitizing agent and more selective ablation of abnormal vessels was a breakthrough, but it was still associated with significant recurrence rates (Azab et al., 2005). A newer understanding of the molecular basis for the development and progression of neovascularization in AMD (Adamis et al., 1994; Aiello et al., 1994; D'Amore, 1994; Frank et al., 1996) was applied to generating effective anti-angiogenic therapies for neovascular AMD in the past decade. Anti-VEGF agents, which can prevent new vessel development rather than only destroying existing vessels, have significantly improved the prognosis of NV-AMD (Schmucker et al., 2012; Kaiser, 2013). For the treatment of NV-AMD, the FDA first approved Pegaptanib (Macugen; Eyetech/Pfizer) (Gragoudas et al., 2004), followed by Ranibizumab (Lucentis; Genentech) (Rosenfeld et al., 2006; Chang et al., 2007; Brown et al., 2009) and Aflibercept (Schmidt-Erfurth et al., 2014), all of which are anti-VEGF agents.

Pegabtanib, a pegylated single-strand nucleic acid that binds to 165 isomer VEGF, was the first FDA approved anti-VEGF drug. Pegaptanib use has declined significantly in recent years because of the development of more effective anti-VEGF agents such as Ranibizumab. Ranibizumab is a humanized, recombinant monoclonal antibody fragment that targets all isoforms of VEGF-A and is administered by intravitreal injection. Two randomized clinical trials, ANCHOR and MARINA, with a follow-up period of 12 and 24 months, validated therapeutic use of Ranibizumab for the treatment of classic and occult CNVs (Rosenfeld et al., 2006; Chang et al., 2007; Brown et al., 2009). In the SEVEN-UP study, an extension for the patients who were originally treated in ANCHOR and MARINA studies, about one third of the patients showed improved visual outcomes and one third showed poor visual outcomes 7 years after the initiation of intensive treatment with Ranibizumab for NV-AMD. About half of the eyes were stable compared to the baseline (Rofagha et al., 2013). Bevacizumab (Avastin; Genentech) is a full-length, humanized, monoclonal antibody that blocks all active isoforms of VEGF-A and is also administered by intravitreal injection although it is not approved by FDA for this application. Clinical cohorts of patients treated with Ranibizumab and Bevacizumab revealed equal efficacy and safety profiles for both agents in the CATT study (Steinbrook, 2006; Group et al., 2011; Comparison of Age-related Macular Degeneration Treatments Trials Research et al., 2012). Bevacizumab has gained popularity primarily because of its substantially lower price (Rosenfeld, 2006; Steinbrook, 2006) and similar clinical effect compared to Ranibizumab (non-inferiority) (Group et al., 2011; Comparison of Age-related Macular Degeneration Treatments Trials Research, Martin et al., 2012).

Aflibercept (Eylea, Regeneron) is a human recombinant fusion protein that consists of the extracellular domain of the VEGF receptors 1 and 2 fused to the Fc portion of IgG1. Aflibercept demonstrates higher affinity to all VEGF isoforms compared to all other anti-VEGF agents, including Ranibizumab and Bevacizumab (Economides et al., 2003; Brown et al., 2011; Heier et al., 2012). Aflibercept, even when administered every 8-weeks by intravitreal injection, is proven equally safe and effective in the treatment of NV-AMD when compared to monthly Ranibizumab injections (Heier et al., 2012).

Emerging treatment modalities include RNA interference and attempts to block pathways down- or up-stream of VEGF to prevent new vessel growth and pericyte recruitment (such as anti-platelet derived growth factor [PDGF] agents). Some of these therapies cause neovascular membrane regression and can potentially improve outcomes when used in combination with anti-VEGF therapies (Hanout et al., 2013; Kaiser, 2013). Anti- PDGF medication (Fovista™, Ophthotech, US), in combination with anti-VEGF medications, prevent PDGF from binding to its natural receptor on pericytes, enhancing the effect of anti-VEGF drugs on new vessel regression. Ophthotech has completed a multicenter, randomized, double-masked, controlled Phase 2 clinical trial evaluating the efficacy and safety of Fovista™ administered in combination with Lucentis™ (Ranibizumab) for the treatment of patients newly diagnosed with NV-AMD (Tolentino et al., 2014). The study demonstrated significant superiority of combination therapy to Lucentis monotherapy in visual acuity gain at 24 weeks. The drug exhibited a good safety profile and no significant safety differences were observed for combination therapy when compared to Lucentis monotherapy. A phase 3 trial is now underway to collect data for FDA approval of the clinical use of the medication.

Although prognosis of NV-AMD has changed dramatically during the last decade because of the availability of anti-VEGF medications, many patients may still lose their vision because of late diagnosis or inadequate treatment. Replacement of disorganized

and lost RPE and PR cells with stem cell-derived RPE or RPC can potentially restore vision in such patients.

4.2. Non-exudative AMD

The molecular mechanisms for NNV-AMD are not fully defined, but there is evidence that more than one pathway is involved in the accumulation of oxidative stress culminating in RPE dysfunction and death. These pathophysiologic mechanisms are reviewed in comprehensive publications (Zarbin, 2004; Ambati and Fowler, 2012; Cascella et al., 2014). Unlike in NV-AMD, where blocking a single culprit molecule (i.e. VEGF) can stop progression of the disease, redundant pathways of NNV-AMD make targeting a single agent unrealistic. Treatment strategies are divided into three major groups: (1) preventing RPE dysfunction or death; (2) providing support to stressed RPE to maintain its function for an extended period; and (3) replacing diseased RPE with a new, healthy RPE layer (to be discussed below). Future successful treatments will most likely be combinations of two or all three of these major group strategies.

5. RPE and photoreceptor replacement approaches to the treatment of AMD

The RPE is responsible for the regeneration of visual photopigments, transport of oxygen and nutrients to photoreceptors, and phagocytosis of photoreceptor outer segments, as mentioned above. The RPE also maintains the subretinal mircoenvironment through production of cytokines and chemokines and forms the outer blood-retinal barrier. Evidence supports the idea that the RPE is the initial site for events leading to AMD as cited above. Therefore, the RPE is considered to be an important target for therapeutic interventions to treat both NNV- and NV-AMD (Binder et al., 2007). Because the RPE is incapable of self-renewal, replacement of diseased RPE with healthy cells was attempted in the 1990s using transplantation of autologous RPE sheets harvested from the retinal periphery (Phillips et al., 2003). Transplantation of autologous extramacular RPE sheets underneath the fovea was associated with visual improvement, providing proof-of-principle that RPE replacement therapy is a potential treatment for dry AMD (Majji and de Juan, 2000; Joussen et al., 2006; Binder et al., 2007). However, the cell sources for autologous transplants are limited and harvesting them is a challenging operation associated with multiple complications (Joussen et al., 2007; Caramoy et al., 2010). In addition, autologous peripheral RPE shares the same genetic defects as the diseased RPE in the macula. Fetal and adult RPE have both been tested as a source of RPE for replacement therapy, but these sources are also limited. In addition, it has been shown that these cells degenerate easily upon implantation in the subretinal space (Algvere et al., 1994). It is thus clear that RPE transplantation therapy needs a more abundant and possibly robust source of healthy RPE to make it a feasible treatment option.

5.1. Potential sources of cells in RPE-photoreceptor replacement therapies

Translocation of autologous RPE sheets from peripheral retina (Fujii et al., 2002) and human fetal retinal and RPE sheets into the subretinal space of patients with macular degeneration has been tolerated well and was associated with improved vision in some cases (Radtke et al., 2008). However, as stated before, these are very limited sources of cells and may themselves contain predisposing genetic defects for continued retinal damage. Introduction of stem cell-derived RPE has opened a new era where a plentiful, well-controlled source of RPE can be available for replacement

therapy. It is very likely that derivation of large numbers of RPE cells from stem cells can provide a commercially viable supply of RPE cells for transplantation therapy while avoiding genetic defects inherent in use of autologous RPE.

5.2. Stem cell-derived RPE (Fig. 2)

Stem cells, by definition, are able to (1) be self-renewing, proliferating and reproducing the same multipotent stem cells indefinitely in their undifferentiated state, and (2) differentiate into different cell types. Commonly used stem cell-related terms are summarized in Table 1.

hESCs are pluripotent stem cells that were first isolated and cultured in 1998 by Thomson et al. (1998). In 2004, Klimanskaya et al. developed the original protocol for hESC-derived RPE-like cells (Klimanskaya et al., 2004). This approach created great hope that a theoretically endless source of RPE cells for the treatment of AMD could be possible. During the past decade, different groups have used several strategies to derive RPE cells from stem cells with variable success. However, many technical and regulatory breakthroughs in the last few years have made stem cell-based treatment for AMD more plausible. These include (1) derivation of RPE cells from hESCs, (2) advances in the use of iPSCs, (3) the use of stem-cell-derived retinal progenitor cells to replace photoreceptor loss, and (4) actual clinical trials using stem-cell-derived-RPE in retinal degenerative diseases. With increasing evidence indicating that short-term safety of stem cell derived-RPE implantation is not a major concern (Schwartz et al., 2015), the remaining major obstacles in the clinical application of stem cell-based therapeutics for AMD are (1) development of commercially viable sources of RPE and retinal progenitors that are low cost, reliable, and robust; (2) optimization of the methods to deliver stem cell derived-RPE into the subretinal space; (3) long-term survival and function of the implanted cells; (4) long-term safety of the procedure, such as the lack of tumor formation years after implantation; and (5) methods to leverage the immunoregulatory potential of stem cell-derived RPE to minimize the risk of immune rejection. In addition, it has yet to be determined whether stem cell-derived RPE or stem cell-derived RPC replacement therapies just slow the progression of retinal degeneration or actually improve vision by integrating into the retina and restoring retinal function.

Transplantation of hESC-RPE into the subretinal space of animal models of retinal degeneration has been shown to rescue the degenerating photoreceptors and improve vision (Lund et al., 2006, 2007; Lu et al., 2009). These promising results have provided the proof of concept that hESC-RPE transplantation may prove a practical treatment option. Optimizing RPE transplantation procedures has resulted in the development of two different therapeutic strategies: (1) introducing a cell suspension of non-polarized hESC-RPE cells into the subretinal space and allowing the donor cells to integrate within the host retina, and (2) transplanting polarized sheets of hESC-RPE to allow for improved safety and better clinical outcomes, since normal RPE function is dependent on specific cellular features of its apical and basal domains. iPSC-RPEs derived from a patient's own cells show genuine RPE cell characteristics including being able to interdigitate with the photoreceptor outer segments and support them (Kamao et al., 2014). Stem cell-derived RPCs have been proposed to replace lost photoreceptors with or without combined hESC-RPE cell transplantation (Luo et al., 2014; Coles et al., 2004). Fetal umbilical cord blood cells and hematopoetic stem cells are multipotent stem cells that are being explored for the treatment of retinal degenerative diseases (Mooney and Lamotte, 2010; Fox et al., 2014); but, as of today, they have been less promising
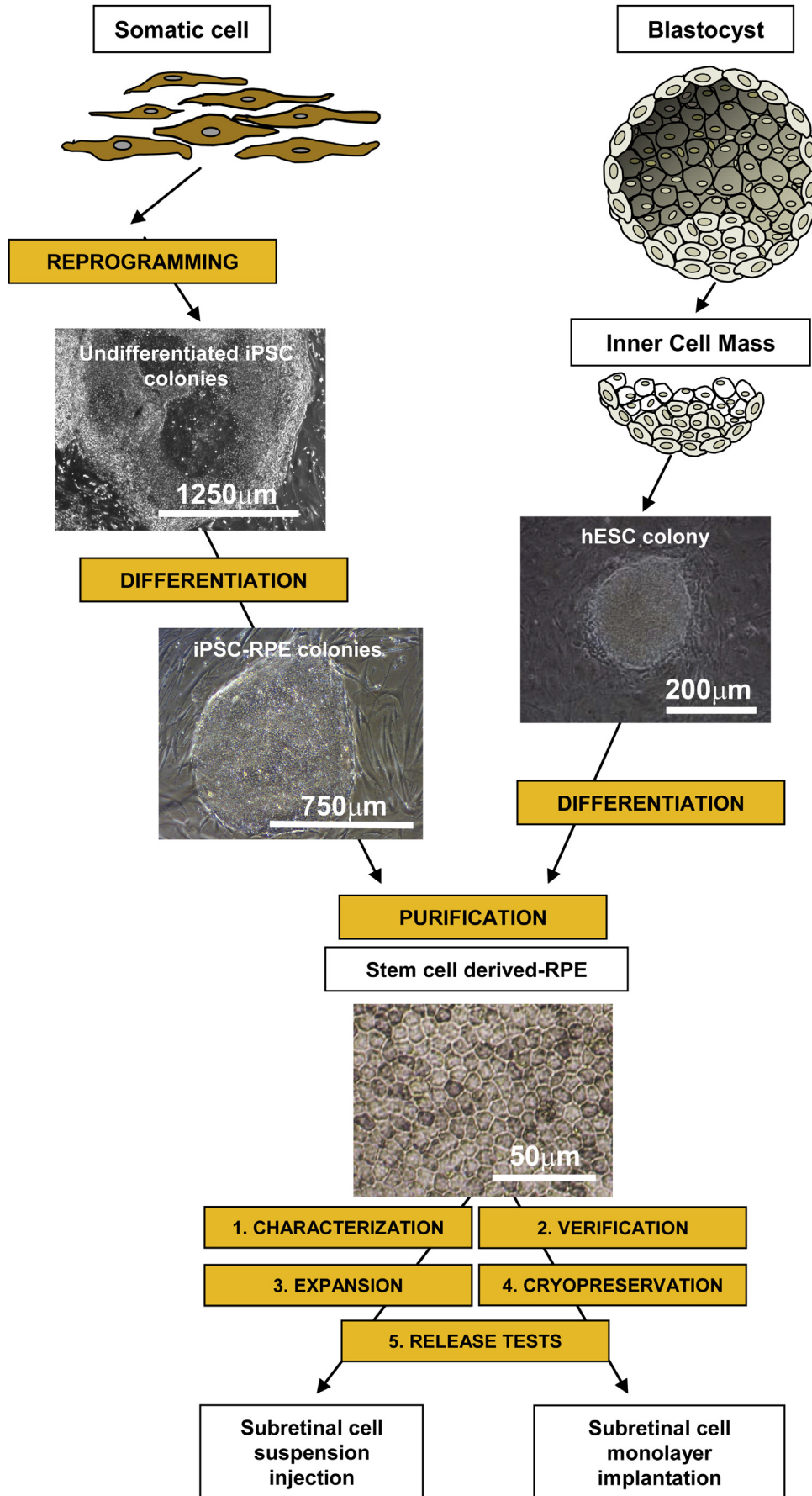
Table 1
Terms and definitions used in the stem cell field.

Term	Definition
Stem cells	Are able to proliferate indefinitely in their undifferentiated state and to differentiate into various cell types.
Omnipotent stem cells	Are capable of forming tissues consisting of embryonic germ layers as well as extra-embryonic tissues.
Pluripotent stem cells	Are able to differentiate into tissues originated from any of three embryonic germ layers (ectoderm, mesoderm, and endoderm).
Multipotent stem cells	Are further advanced in the differentiation ladder, i.e. they are able to develop into limited cell types usually with a single germ layer origin.
Embryonic stem cells (ESC)	Are obtained from a 5-day old blastocyst and are considered to be pluripotent.
Induced pluripotent stem cells (iPSC)	Differentiated somatic cells altered to be pluripotent through dedifferentiation and reprogramming methods.
Mesenchymal stem cells	Multipotent stromal cells that are housed in bone marrow and other tissues and can differentiate into osteoblasts, chondrocytes, and adipocytes.
Somatic stem cells	Stem cells normally present in various adult tissues and supposedly responsible for replacing lost cells.

compared to use of hESC-RPE, iPSC-RPE, and RPC. The following sections describe hESC-RPE, iPSC-RPE, and SC-RPC derivation and their characteristics in more detail.

5.2.1. hESC-RPE

Embryonic stem (ES) cells are derived from blastocysts and share many characteristics with the cells from the epiblast (Thomson et al., 1998). They have the ability to proliferate indefinitely and, because they are considered to be pluripotent, they can differentiate into any of the cell types of all three germ layers (ectoderm, mesoderm, and endoderm). Kawasaki et al. first reported differentiating RPE-like cells from ES cells in 2002, specifically using monkey ES cells (Kawasaki et al., 2002). To generate the RPE-like cells, the monkey ES cells were cultured in the presence of PA6 stromal cells (a bone marrow-derived mouse cell line) in a differentiating medium consisting of Glasgow Minimum Essential Medium (GMEM) medium supplemented with 10% knockout serum replacement/1 mM pyruvate/0.1 mM nonessential amino acids/0.1 mM 2-mercaptoethanol. After about 3 weeks, pigmented colonies of hexagonal cells appeared in a subset of cultures (Kawasaki et al., 2002). The authors reported that these ES-derived RPE-like cells expressed typical RPE markers, such as ZO-1, RPE65, CRALBP, and Mertk, had extensive apical microvilli, and were able to phagocytize latex beads. The cells enhanced photoreceptor survival upon transplantation into the subretinal space of Royal College of Surgeons (RCS) rats, a well-established model of AMD (Amirpour et al., 2012). Haruta et al. (2004) had originally reported successful transplantation of ES-derived RPE cells into the RCS rat subretinal space with enhanced survival of host photoreceptor cells (Haruta et al., 2004). Variable culture protocols have since been explored for efficient and rapid in vitro differentiation of RPE from ES cells lines derived from other animals (Osakada et al., 2008; Meyer et al., 2009; Osakada et al., 2009a, 2009b; Amirpour et al., 2013).

5.2.1.1. Differentiation methods

5.2.1.1.1. Spontaneous differentiation of hESC into RPE. The spontaneous differentiation of human RPE cells from hESCs is described in multiple publications (Hirano et al., 2003; Klimanskaya et al., 2004; Lund et al., 2006). hESC-RPE cells were initially obtained by culturing hESCs on inactivated mouse embryonic fibroblasts (MEFs) and then removing basic fibroblast growth factor (bFGF) from the culture medium; bFGF is necessary for keeping hESCs in an undifferentiated state (Klimanskaya et al., 2004). Since then, it has been shown that, with bFGF deprivation, RPE-like cells can be derived through culture on various substrates other than MEFs, including the feeder-free substrate Matrigel, poly-D-lysine-coated dishes, and laminin (an extracellular matrix protein) (Rowland et al., 2013). hESC-RPE derivation has also been successful using various hESC culture media, such as DMEM/F12 media with knockout serum replacement (KSR), mTeSR1 media (StemCell), and XVIVO 10 media (Lonza). The spontaneous differentiation process takes about 10—12 weeks and results in a large number of pigmented colonies of hexagonal RPE-like cells expressing RPE-specific genes. Typically, after 10—12 weeks of spontaneous differentiation, there are many small pigmented spots (i.e., RPE cell like colonies) that are then manually microdissected to generate an enriched culture of RPE that is >99% pure. Modifications in defined factors at specific times have also resulted in more efficient differentiation of hESC-RPE cells (Buchholz et al., 2013).

5.2.1.1.2. Two-stage induction of RPE from hESC. Because RPE cells are differentiated from the neural ectoderm and share common characteristics with neuronal retinal cells in vivo, a related two-stage induction procedure was investigated for its ability to produce RPE cells from neural retinal precursors (Cho et al., 2012; Lamba et al., 2010; Zhu et al., 2011; Zhu et al., 2013). Specifically, hESC aggregates were initially cultured in suspension with neuronal differentiation media to induce differentiation into neural precursors. The neural precursors were then allowed to expand and differentiate into putative RPE-like cells. During these stages of induction and differentiation, the putative RPE cells appeared as early as 4 weeks and reached large numbers of cells suitable for subculture at approximately 8 weeks. This two-stage method has the advantage of being more time and labor efficient compared to the spontaneous derivation method.

5.2.1.1.3. Forced induction of hESC to RPE. Based on the role of nicotinamide (NIC) in cell metabolism, survival, plasticity, and differentiation, Idelson et al. investigated the effect of NIC on the differentiation of hESCs to RPE cells (Idelson et al., 2009). They first developed cell clusters from hESCs using collagenase and, to induce RPE differentiation, they cultured the clusters in suspension with hESC culture media supplemented with 14% KSR, NIC, and with or without Activin A (a TGF-β superfamily member that is believed to direct differentiation of optic vesicle to RPE in the embryo (Fuhrmann et al., 2000). While NIC blockage decreased the percentage of pigmented cell colonies to less than half, addition of

Fig. 2. Flowchart showing derivation of human embryonic stem cell-derived retinal pigment epithelium (hESC-RPE) and induced pluripotent stem cell-derived retinal pigment epithelium (iPSC-RPE) and preparation of these RPE cells for transplantation. (iPSC and iPSC-RPE colonies are reproduced with permission from John Wiley and Sons publication: Qirui Hu, Amy M. Friedrich, Lincoln V. Johnson, Dennis O. Clegg. Memory in induced pluripotent stem cells: reprogrammed human retinal-pigmented epithelial cells show tendency for spontaneous redifferentiation. Stem Cells 28(11), 2010, 1981—1991; hESC colony figure is reproduced with permission from Schulz TC, Palmarini GM, Noggle SA, Weiler DA, Mitalipova MM, Condie BG. Directed neuronal differentiation of human embryonic stem cells. BMC Neurosci 4, 2003, 27).

Activin A to the culture medium containing NIC increased pigmented cell colonies from 20% to 50% (Idelson et al., 2009). Pigmented areas appeared at 4 weeks post-induction, and approximately half of the clusters cultured in media supplemented with both NIC and Activin A were pigmented. The authors suggested that NIC, in the presence of Activin A, efficiently induces and augments hESC-RPE differentiation.

5.2.1.1.4. Rapid differentiation of RPE from hESC. The current methods suffer from major shortcomings including being cumbersome, having a prolonged differentiation process, and inability to obtain a pure hESC-RPE cell population (Buchholz et al., 2013). Buchholz et al. reported a protocol for more rapid and efficient differentiation of hESC-RPE through the combined use of retinal inducing factors (IGF1, Noggin, Dkk1, and bFGF) and other factors (nicotinamide, Activin A, SU5402, and vasoactive intestinal peptide [VIP]), adding these factors at appropriate, different times (Buchholz et al., 2013). They reported that pluripotent stem cells could be directed to RPE cells with an efficiency of 80% as early as 14 days after the onset of differentiation. The authors suggested that this protocol could be useful for relatively quick production of large quantities of RPE for transplantation, as well as for the study of RPE development.

5.2.1.2. Enrichment and purification of stem cell-derived RPE. RPE-like cells can be identified and isolated from other differentiating cells within a stem cell culture plate because of their unique pigment content, hexagonal shape, and monolayer patch growth. Two enrichment methods are described below that may ensure clinical grade purification of stem cell-derived RPE during the derivation procedure:

One enrichment method utilizes mechanical excision of differentiated RPE patches: After RPE-like cells grow to patches about 1 mm in diameter, the pigmented colonies are mechanically dissected with a 31-gauge needle or 25-gauge ophthalmic surgical knife under a dissecting microscope and then transferred into a trypsin-EDTA solution for cell dissociation. The dissociated RPE-like cells are then seeded on Matrigel or gelatin-coated culture dishes for further growth. Although gentle manual picking of early pigmented colonies can reduce the proportion of contaminating non-RPE cells; culturing and expansion of colonies of pure hESC-RPE is still an unresolved challenge (Bharti et al., 2011).

The second method involves enzymatic isolation of differentiated RPE patches from culture: This method consists of two consecutive trypsin digestions to isolate putative RPE cells. It is based on the fact that RPE cells attached to the culture dish surfaces more tightly than other, non-RPE-like cells. During the first trypsin digestion, most of the non-RPE-like cells are dissociated and discarded. A second, longer incubation with trypsin dissociates the RPE-like cells from the culture dish surface. The dissociated RPE-like cells are then seeded on Matrigel or gelatin-coated dishes for further culture.

Using either enrichment method, the newly dissociated RPE cells lose their cobblestone morphology and pigment. However, once the cells grow to confluence within 2–3 weeks, the cells regain their typical RPE morphology and pigmentation. With this plasticity, RPE cells can be passaged 5–7 times with alternative dedifferentiation and differentiation cycles to reach a larger quantity of cells for therapeutic purpose. In general, the mechanical method produces an almost pure population of stem cell-derived RPE cells with fewer non-RPE cell contaminants than the enzymatic isolation method but it is time-consuming and results in lower-cell-yield.

5.2.1.3. hESC-RPE and iPSC-RPE characterization. Commercial production of stem cell-derived RPE implants should be rigorously controlled by developing standard operating procedures (SOPs) for pre-clinical testing for all steps of production to ensure that the cells exhibit the critical quality attributes of clinical-grade RPEs. As the final product is a viable cell that cannot be sterilized or directly tested for genetic signature, purity, consistency, stability, and function, it is imperative to develop and adhere to a series of strictly defined regulatory standards. Automated and semi-automated production of the cells can be used to minimize any human-induced variability. For example, the International Organization of Standardization certified WAVE Bioreactor is a single-use closed bioreactor system used for the production of clinical-grade T cells and NK cells, and in the Bioreactor, these cells are automatically tested for cytotoxicity, local and acute systemic toxicity, irritation and sensitization, endotoxin, and sterility. Such a system could be adopted for producing stem cell-derived cell products like hESC-RPE and iPSC-RPE (Bharti et al., 2014, 2011; Eibl and Eibl, 2009; Timmins et al., 2009). A thorough discussion of regulatory and logistic issues of the production of stem cell-derived RPE implants has been published by Bharti et al. (2014).

In order to refine stem cell-derived RPE differentiation methods and to control the quality of clinical-grade stem cell-derived RPE cells, a standardized and well accepted set of physiologically-relevant morphologic, molecular, and functional signatures should be defined and used as the release criteria for the RPE cells (Table 2) (Maminishkis et al., 2006; Carr et al., 2009a, 2009b; Bharti et al., 2011). The stem cell-derived RPE should replicate fetal RPE in gross and ultrastructural morphology and in genetic and functional features. Based on those RPE characteristics, the following described methodologies can be used for in vitro verification and identification of stem cell-derived RPE cells.

5.2.1.3.1. Verification of RPE morphology. The unique cobblestone (or hexagonal) morphology and dark brownish pigmentation of differentiated, polarized RPE cells can be easily observed under a light microscope (Fig. 3A). Stem cell-derived RPE monolayers should be composed of cells that are phagocytic, polygonal, pigmented, and polarized i.e., where intracellular organelles are organized in a highly polarized way, similar to the apical-basal orientation of RPE cells *in vivo*. Based on ultrastructure analysis at the electron microscope level, polarization of stem cell-derived RPE monolayers should be evident by their having prominent apical microvilli and basally localized hemidesmosomes and melanin pigments (Fig. 3B and C). Staining for the RPE-specific marker, RPE65, and markers showing specific secretory function of RPE cells, such as PEDF and VEGF, would confirm the differentiated cell type (Fig. 3D and E). In addition, hESC-RPE cells should form tight junctions to become polarized (Fig. 3F and G). The polarization of these cells can be further characterized by investigating the presence of apically distributed sodium/potassium ATPase (Na^+/K^+ ATPase) channels and intercellular tight junctions. Tight junction-specific proteins (such as occludin and ZO-1) (Fig. 3F and G) and Na^+/K^+ ATPase channels can be examined using immunocytochemistry (Rizzolo, 1990; Nabi et al., 1993; Sonoda et al., 2009).

5.2.1.3.2. Identification of RPE specific genes. The expression of RPE specific genes (signature genes), including (1) vision cycle genes such as *RPE65*, *RDH 11*, and *CRLBP*; (2) RPE membrane channel and transporter genes such as *BEST1* and *SLC*; (3) pigment biosynthesis and melanin biosynthesis genes such as *GPR143*, *TYRP1*, dopachrome tautomerase [*DCT*], *SILV*, and *MITF*; and (4) phagocytic activity genes such as *LAMP2*, *VDP*, *Mertk*, and *GULP1*, have been used to characterize stem cell-derived RPE cells (Lamba et al., 2010; Liao et al., 2010; Strunnikova et al., 2010; Vugler et al., 2007). While there is no universally agreed upon minimum number of signature genes to identify the cells as "RPE," we test our cells at least for RPE65, BEST1, and MITFiso5 before releasing them as hESC-RPE (Fig. 3).

5.2.1.3.3. Search for genes of contaminating cell types. As the

Table 2
Assays used to characterize stem cell-derived RPE (adopted and summarized from Bharti et al., 2011).

	RPE characteristic	How to characterize	Reference
1	RPE cell morphology	Visual inspection (via light microscope) of pigmented monolayer of confluent hexagonal epithelial cells.	(Sonoda et al., 2009)
2	RPE signature genes	Analysis of gene and protein expression of markers involved in melanognesis, channel proteins, intercellular tight junctions, the visual cycle, response to sensory perception, oxidoreductase activity, and phagocytic and transporter activity	(Strunnikova et al., 2010)
3	Absence of pluripotency and non-epithelial marker expression	Analysis of gene and protein expression of markers associated with early development of pluripotent stem cells and neuroextodermal cells of the optic cup such as OCT4, SOX2, KLF4, NANOG, MYC, PAX6, MITF, LIN28. Analysis of expression of epithelial–mesenchymal transition markers such as ZEB1, TGFβR.	(Carr et al., 2009a, 2009b)
4	RPE polarization	The presence of tight junctions allowing transepithelial resistance increase up to several hundred $\Omega.cm^2$ measurement of apical to basolateral membrane fluid absorption across the monolayer	(Sonoda et al., 2009)
5	Vectorial secretion of cytokines and growth factors	Apical or basal distribution of surface molecules including channels, receptors, transporters proteins. Predominantly apical secretion of growth factors and cytokines such as PDGF, TGF-β2, IL-6, and IL-8. Predominantly basal secretion of growth factors and cytokines such as VEGF, IL-6, IL,8, MCP-1, ITAC, RANTES, IP-10.	(Zhu et al., 2011) (Hirsch et al., 2015)
6	Phagocytosis of photoreceptor outer segments	Labeled bovine and human POS could be traced inside the hESC-RPE (this phagocytosis is dependent on MERTK expression). Polystyrene beads phagocytosis (not dependent on MERTK expression)	(Kevany and Palczewski, 2010) (Carr et al., 2009a, 2009b)
7	Immunologic properties of RPE cells	Visual inspection of polarized secretion of cytokines including PDGF, TGF-β2, VEGF.	(Zhu et al., 2011) (Hirsch et al., 2015)

purity of clinical-grade, stem cell-derived RPE cells is vital for cell replacement therapies, RPE cell cultures should be tested for potentially contaminating cells through expression analysis of specific genes, including (1) undifferentiated stem cell markers such as *Oct4*, Lin28, and *REX1)*; (2) neural cell markers such as *MAP2*; (3) fibroblast markers such as *S100A4*; (4) endothelial marker *PECAM1*; and (5) non-RPE melanocyte marker *MITF iso4/5* (Amae et al., 1998; Ben-Shushan et al., 1998).

5.2.1.3.4. Confirmation of RPE function. Among many functional properties of mature RPE cells, phagocytosis of photoreceptor outer segments is considered a benchmark. Under culture conditions, the phagocytosis ability of hESC-RPE can be confirmed by the ability to phagocytize latex beads (Klimanskaya et al., 2004; Osakada et al., 2008) or FITC-labeled rod outer segments (ROS) (Fig. 3H and I) (McConnell, 1965; Papermaster and Dreyer, 1974). Likewise, intracellular rhodopsin immunostaining is used to ensure that hESC-RPE cells are able to actually phagocytize photoreceptor outer segments after being implanted in the subretinal space of test animals (Fig. 3) H and I.

5.2.2. iPSC-RPE

The successful derivation of adult cells from a mammalian egg, first reported in sheep using somatic cell nuclear transfer (introducing a somatic cell nucleus into an unfertilized, enucleated egg) (Wilmut et al., 1997), has suggested that embryonic cells contain factors that can confer totipotency or pluripotency to somatic cells (Takahashi and Yamanaka, 2006). Takahashi and Yamanaka demonstrated that forcing mouse fibroblasts to express four factors, Oct3/4, Sox2, c-Myc, and Klf4 (but not Nanog), induces the cells to become pluripotent stem cells. The so-called induced pluripotent stem cells (iPSCs) express ESC markers, and exhibit the morphology and growth properties of ESCs. Subcutaneous transplantation of iPSCs into immunosuppressed mice resulted in tumors containing tissues from all three germ layers (Takahashi and Yamanaka, 2006), demonstrating pluripotency and iPSC's capability of evading immune detection.

The derivation of pluripotent stem cells from somatic cells has been promising for the production of patient-specific cells with theoretically no risk of immune rejection (Takahashi and Yamanaka, 2006; Fusaki et al., 2009). iPSCs share the self-renewal and pluripotency characteristics of hESCs and thus are another source for differentiated cells for use in regenerative medicine, disease modeling, drug testing, and exploring developmental

biology. Because iPSCs can be directly generated from theoretically any adult tissue, each patient could have their own iPSCs, offering a unique cell source for replacing a degenerated or lost organ with theoretically zero chance of immune rejection. To develop AMD treatments, human iPSCs have been differentiated into RPE cells. Although some groups have shown that hESC-RPE resembles human fetal RPE more closely than iPSC-RPE (Liao et al., 2010), iPSC-RPE cells have been shown to have a full range of morphologic and functional properties characteristic of RPE cells in vitro and *in vivo* (Li et al., 2012). These iPSC-RPE cells are morphologically similar to, and express key markers of, developing and mature RPE cells (Hu et al., 2010; Buchholz et al., 2009; Carr et al., 2009a, 2009b). Transplantation of iPSC-RPE cells into RCS rats containing the MERtK gene defect rescues vision in those animals, although cell survival and safety issues are still major concerns for clinical use of iPSC-RPE (Maeda et al., 2013). In addition, iPSCs derived from an AMD patient would still carry genetic abnormalities and senescence changes predisposing them for age-related diseases (including AMD) and consequently may not be suitable for therapeutic use.

In addition to being used for therapeutic purposes, iPSCs are an excellent source of cells for disease modeling and drug development studies. Multiple disease models have been generated from iPSCs, such as diabetes models, liver disease models, and neurodegenerative disorder models (Rashid et al., 2010; Chamberlain et al., 2008; Maehr et al., 2009). Recently, Jin et al. generated a retinitis pigmentosa (RP) cell model from iPSCs by initially deriving iPSC lines from the fibroblast cells of five RP patients with distinct mutations in the *RP1, RP9, PRPH2,* or *RHO* genes. The iPSCs were then differentiated into rod photoreceptor cells (Jin et al., 2011, 2009) in which markers of oxidative and endoplasmic reticulum stress were studied (Lustremant et al., 2013).

5.2.3. Stem cell-derived photoreceptor cells

Currently, no therapy exists to repair or restore damaged retinal cells and vision. Subretinal transplantation of dissected sheets of fetal-derived retinal progenitor cells, combined with RPE, has showed successful anatomic integration and rescue of functional phenotype in animal and human studies (Seiler and Aramant, 2012). Although deriving multilayered retina tissue (with its unique structural complexity) from pluripotent stem cells is a significant challenge, attempts to develop retinal progenitor cells with the ability to incorporate into the existing retinal layer and further
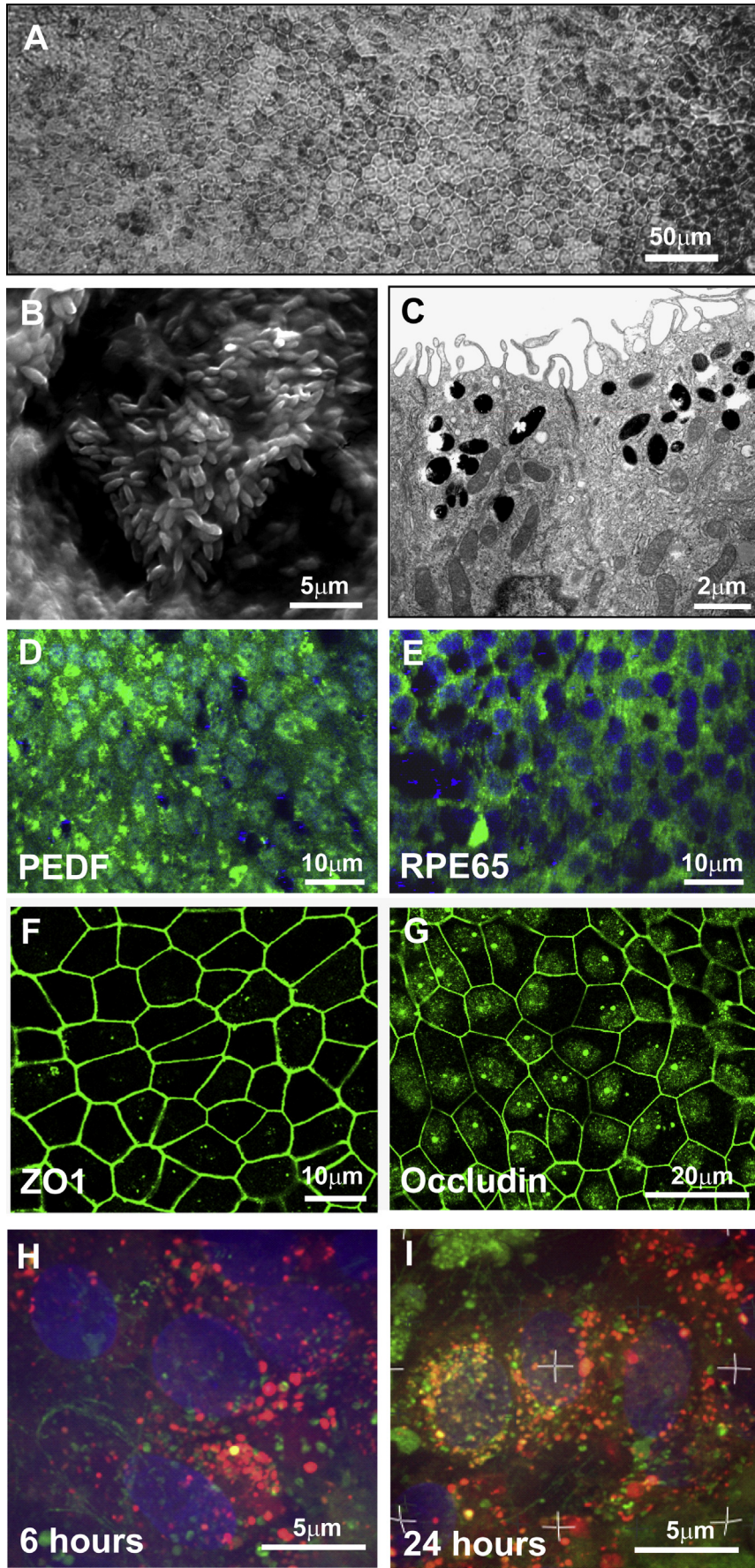
differentiate in situ have been promising. Photoreceptors and their progenitor cells (RPCs) have been derived from various stem cells, including embryonic or fetal retinal progenitors, neurospheres, neural stem cells, and iPSCs, in vivo and in vitro (Nishida et al., 2000; Coles et al., 2004; Klassen et al., 2004; Zhang and Ding, 2011; Luo et al., 2014). Several groups have transplanted various types of RPCs, originating from mouse photoreceptor precursors (MacLaren et al., 2006), rat retinal progenitor sheets (Seiler et al., 2010), or hESC-derived photoreceptors (Lamba et al., 2009), into the vitreous cavity or subretinal space of animal models with degenerative retinal disease and have shown that RPCs could migrate into the retina, differentiate into photoreceptor cells, and ultimately result in anatomic and functional rescue of the degenerating retina (Aftab et al., 2009; Luo et al., 2014).

Reh et al. first reported the successful differentiation of hESCs into retinal progenitor cells using a combination of noggin, dkk1, and IGF-1 (Lamba et al., 2006). However, these progenitors primarily differentiate into inner neuron cells and rarely into photoreceptors, unless they are cultured with embryonic retinal tissues. Further differentiation into photoreceptors from hESC-RPC was studied by Osakada et al. (2008). They cultured feeder- and serum-free hESC suspensions in media supplemented with Wnt and Nodal inhibitors to induce differentiation of Rx + or Mitf + retinal progenitors, which they further differentiated into hESC-RPE cells. They also differentiated the Rx + or Mitf + retinal progenitors into photoreceptors by subsequent treatment with retinoic acid and taurine (Osakada et al., 2008; Osakada et al., 2009). More recently, Assawachananont and co-workers have reported successful transplantation of embryonic and iPSC-derived 3D retinal sheets into a retinal degenerative mouse model lacking a structured outer nuclear layer (Assawachananont et al., 2014).

hESC-derived RPC (hESC-RPC) cell lines express markers specific for neural progenitors, including the intermediate filament nestin, the microtubule subunit β3-tubulin, the glial marker vimentin, and SSEA4, a pluripotency stem cell marker. These cells also express transcription factors related to eye development, including PAX6 and NeuroD1 (Luo et al., 2014). When induced to differentiate in vitro, hESC-RPCs differentiate into cells expressing photoreceptor-specific transcription factors such as CRX, OTX2, and NRL and other photoreceptor-specific markers such as recoverin. Quantitative flow cytometry analysis and gene expression analysis confirmed the expression of early eye field transcription factor genes such as PAX6, LHX2, and SIX6 (Lamba et al., 2010; Luo et al., 2014). Furthermore, it has been shown that the expression of retinal progenitor markers in hESC-RPCs can be maintained over multiple passages in culture while the expression of pluripotency markers, including NANOG and OCT4, significantly decreases. hESC-RPCs also express increased levels of growth factors, such as brain-derived neurotrophic factor (BDNF) and fibroblast growth factor 2 (FGF2), compared to the adult retina. On the other hand, the expression of growth factors such as TGF-β2, which suppresses mitotic activity and acts as a cytostatic factor on adult neurogenesis in the brain, is decreased (Luo et al., 2014). The higher levels of neurotrophic agents, such as BDNF, FGF2 and lower level of mitotic factor, TGF-β, secreted by hESC-RPC may exert their effect by rescuing host photoreceptors and modifying host responses. The RPCs are also capable of potentially improving visual acuity, as

when RCS rats underwent subretinal injection of RPCs (Luo et al., 2014). In these studies, the rats showed significantly better visual acuity compared to non-treated and vehicle-treated eyes at 12 weeks post-injection. Lund's group recently reported subretinal injection of human neural progenitor cells under the retina of RCS rats (Gamm et al., 2007; Wang et al., 2008). At day 100, when no visual function can normally be detected in untreated RCS rats, treated rats exhibited substantial retinal electrical activity and retained visual field and visual acuity (Gamm et al., 2007). Furthermore, histologic evidence has shown that human neural progenitor cells could survive for prolonged periods, migrate extensively, and secrete growth factors in subretinal space of RCS rats and significantly better preserved outer nuclear layers (ONL) in transplanted areas (Gamm et al., 2007) (Luo et al., 2014; Guo et al., 2003).

The intended beneficial effect of RPCs is likely accomplished through differentiation to functioning retinal cells and taking over lost or dysfunctional elements (Warfvinge et al., 2005; Klassen et al., 2007). Because survival of ONL cells was observed to extend beyond the area engrafted with hESC-RPCs, it is likely that the survival of cell is not achieved only through cell replacement but also through trophic factors produced by RPCs as suggested in multiple studies (Lund et al., 2001a, 2001b; Lund et al., 2007; Lu et al., 2010; Luo et al., 2014). For example, in one study, RCS rats engrafted with hESC-RPC, double labeling of grafted cells with photoreceptor markers (including recoverin, opsin, rhodopsin) and a human-specific anti-nuclei antibody was performed and failed to show differentiation of donor cells into mature photoreceptor cells (Luo et al., 2014). This may indicate that the observed rescue effect was because of trophic factors secreted by the injected hESC-RPCs. Supporting the notion that paracrine secretion of growth factors can actually rescue remaining retinal elements, it has been shown that intraocular delivery of growth factors retards the progression of retinal degeneration, both in animal models of retinal degeneration and in patients with AMD (Frasson et al., 1999; Zhang et al., 2011). All of these experiments have been carried out with xenographic RPCs; whether an allograft of RPCs can better integrate into the retina and replace the lost cells has not been investigated.

A major hurdle in incorporating photoreceptor progenitors to the retina is to establish that they do, in fact, make functional synaptic connections with the proximal neuronal elements. A milestone was reached with the differentiation of hESCs into optic cups showing stratified neural retina structure. Sasai's group developed mouse retinal anlages in vitro by using a relatively simple 3D culture method and demonstrated that the optic cup morphogenesis depends on an intrinsic self-organizing program involving stepwise and domain-specific regulation of local epithelial properties (Eiraku et al., 2011; Nakano et al., 2012). The self-formation of fully-stratified 3D neural retina tissues may herald the next-generation of regenerative medicine in retinal degeneration therapeutics where the major emphasis would be on building connections to ganglion cells/optic nerve axons.

6. Clinical grade manufacturing

Delivering a cell-based therapy originated from stem cells involves unique regulatory and quality control challenges. Such

Fig. 3. Cellular morphology, marker expression, and phagocytic function of human embryonic stem cell-derived RPE (hESC-RPE). (A) Pigmented, hexagonal hESC-RPE cell monolayer visualized using a light microscope. (B) Scanning electron microscope (SEM) and (C) transmission electron microscope (TEM) micrographs of hESC-RPE revealing ultrastructure features. Photoreceptor outer segments are surrounded by RPE cell's apical side cilia (seen in B and C) where shed outer segment discs are engulfed and phagocytosed into the RPE cells. Immunocytochemistry staining for RPE-specific molecules (green; nuclei labeled in blue), specifically (D) PEDF, (E) RPE65, (F) ZO1 and (G) occludin. (H) Phagocytosis of FITC-labeled bovine photoreceptor outer segments (green) by hESC-RPE after 6 hours or (I) 24 hours of incubation. (Lysosomes are stained in red, phagolysosomes in yellow, and nuclei in blue.) (Figures reproduced with permission from: Zhu D1, Deng X, Spee C, Sonoda S, Hsieh CL, Barron E, Pera M, Hinton DR. Polarized secretion of PEDF from human embryonic stem cell-derived RPE promotes retinal progenitor cell survival. Invest Ophthalmol Vis Sci 52(3), 2011, 1573—1585).

Table 3
Quality control during the production of clinical grade stem cell-derived RPE graft.

Safety issue	How to address
Sterility	Sterile condition during the entire development and delivery of stem cell-derived RPE cells.
	Cell batches in development process are checked for Mycoplasma contamination.
Purity	Meticulous manual picking of putative RPE colonies during the differentiation process
	Visual confirmation of uniform cobblestone morphology
	Cells are checked for non-RPE gene expressions
Misdifferentiation	Cells are checked for non-RPE gene expressions
Tumorigenicity	Transplantation of stem cell-derived RPE cell from the original cell bank into subretinal space of nude rats.
Immunogenicity	Co-cultured with peripheral blood monocytes
	Serum cytotoxicity assays

therapeutic cells should have optimum safety, identity, purity, and potency before being transplanted into the targeted organ (Table 3). The stem cells must be maintained, differentiated, stored, and recovered under valid current good manufacturing practice (GMP). The differentiated final product must be free of pathogens and undifferentiated cells. The cells must possess the appropriate characteristics of fully functional differentiated cells. This is usually achieved through extensive preclinical tests in animals for the absence of teratomas and migration of cells into other organs, and to ensure the proper function of the cells.

NIH Drug Master Files (DMF) that contain complete information on the hESC/iPSC manufacturing process and preclinical validation is recommended as a reference to other academic and private groups interested in such efforts. In addition, efforts for "intellectual synergy and cost reduction that benefit the biomedical community and ultimately, the American public" should be sought by public and private sectors (Bharti et al., 2011). An in depth discussion about a scientifically proven set of regulatory and quality control criteria for stem cell-derived RPE/RPC therapies is beyond the scope of this review.

7. Immunobiology of stem cell-derived RPE and RPC

While general concepts of graft immune rejection apply to stem cel-derived tissue replacements as well, individual immune properties of the stem cell-derived tissues and the environment where they are intended to repair/regenerate are of particular importance. Host immune reaction against the implanted cells is a major concern for allogeneic grafts (i.e. tissues from a genetically dissimilar individual although within the same species). Thomson et al. in their original paper describing hESC derivation proposed three strategies to prevent immune rejection of the transplanted stem cell-derived cells: (1) banking ES cells with known major histocompatibility complex antigens and using them for recipients with the closest MHC antigen composition, (2) manipulating hESCs' genetic contents to reduce the chance of rejection, or (3) actively suppressing the host's immune system after the transplantation (Thomson et al., 1998). Using hESC-RPE and iPSC-RPE cells necessitates additional considerations based on the unique immune properties of RPE cells and the subretinal space. The potential for the stem cell-derived RPE cells to replace degenerated endogenous cells in AMD and other degenerative diseases, and to restore lost vision, is challenged by the threats to the implanted cells by the recipient's immune system. Although immune suppressive agents have been used to address the rejection, significant morbidity is associated with such therapeutics, especially in the elderly. First, the basic concept of graft immune rejection is reviewed below to refresh our knowledge of what is needed to understand immune activation against implanted SC-RPE cells in the subretinal space. Then, immune consideration, specifically for RPE and the subretinal space, will be discussed.

7.1. Transplantation immunology

Here, we will divide the immune system into innate and adaptive components to best explain the sequence of events in any immune encounter. While not totally separated, mechanistic differences between the innate immune system (mainly reacting against the nonspecific epitopes of foreign particles without having an immunologic memory after the first encounter) and the adaptive immune system (where T-cells recognize specific non-self epitopes and mount increasing cellular and humoral responses against them) requires the development of different clinical approaches to deal with each system in transplantations (Murphy et al., 2011).

The first encounter for any allogeneic (genetically non-identical) cell upon transplantation in the body is with the components of the innate immune system. During the transplantation procedure (regardless of being autologous or from an allogeneic source), contaminating microbial products (e.g., lipopolysaccharide [LPS]) and endogenous proinflammatory factors (e.g., TNF and IL-1beta) are released with mechanical and ischemic injury. Humoral components of the innate immune system, such as complement proteins, and cellular components of the innate immune system, such as natural killer cells and dendritic cells, become activated after encountering these microbial products and proinflammatory factors. Not only does this activation counteract the allogeneic cells, but the innate immune activation also initiates the adaptive immune response to allograft rejection (Murphy et al., 2011). In addition, the innate immune system's natural killer cells (NK cells), activated through stress-induced ligands and inhibited by the expression of MHC class I molecules on the foreign cells, play a major role in transplant rejection as well (Bix et al., 1991).

Differences between the donor's and recipient's cell surface proteins are the primary factor for inciting an adaptive immune response against the allogeneic grafts. Surface molecules that determine survival of implanted cells are called histocompatibility antigens. Histocompatibility antigens are classified into major histocompatibility complex (MHC) antigens, minor histocompatibility complex antigens, and ABO antigens. A simplified, yet practical, description of graft rejection is that it is primarily initiated by the recognition of the graft's major MHC antigens by the recipient's T cells (Lechler et al., 2005), and the T-cells subsequently promote a cellular and humoral immune cascade. Under physiologic conditions, MHC present antigens to circulating T cells. T cells recognizing self-antigens are normally depleted in the thymus during fetal development so that the remaining, circulating T cells are only capable of responding to non-self antigens (of allogeneic cells) presented via MHC. Allogeneic cells are either immediately recognized by T cells that directly recognize allelic variants of MHCs or through a more chronic process that involves processing the donor MHC antigens by the recipient's professional antigen-presenting cells (APC). The recipient's professional APCs, such as dendritic cells and macrophages, take up donor cells and their debris and subsequently present alloantigens to the recipient's effector T cells,

which results in the expansion of allospecific cytotoxic T cells and the generation of alloantibodies through B-cell activation (Bolton et al., 2008). Although all non-self proteins with structural differences from the recipient's proteins can initiate this immune reaction, the main body of reactive T cell responses are initiated by, and aimed at, MHC antigens, partly because of the extreme polymorphism in MHC (Drukker, 2008). T cell priming by professional APCs occurs in regional lymph nodes, where alloantigens from apoptotic and necrotic donor cells taken up by the APCs are specifically presented to CD4 and CD8 T cells. The expression of different costimulatory alloantigens on APCs can be received as either activation or inhibition signals by the reactive T cells (Bour-Jordan et al., 2011). Targeting these stimulatory or inhibitory signals is one of the novel approaches used in preventing graft rejection. In the future, immunomodulatory agents that target costimulatory molecules (e.g. Belatacept, a B7-specific fusion protein that inhibits the CD28 interaction with CD80 and CD86) may play a role in an immunosuppressive regimen that follows RPE transplantation (Webber et al., 2011).

Non-professional APCs, such as donor endothelial cells and grafted RPE, are shown to present alloantigens to recipient T cells too, stimulating their activation and proliferation (Loewendorf and Csete, 2013). After interferon gamma-stimulated expression of MHC and costimulatory molecules (such as CD80) on donor endothelial cells, these cells can present alloantigens to recipient T lymphocytes and stimulate reactive CD4 and cytotoxic lymphocytes (Loewendorf and Csete, 2013). hESC-RPE may also have the same potential to interact with recipient lymphocytes by MHC expression and presenting alloantigens to them.

While an innate immune reaction composed of complement proteins reacts immediately to alloantigens, priming and expansion of the adaptive immune reaction as discussed above may take up to a few weeks. A second exposure to the alloantigen can spark an acute or hyperacute immune reaction by the preformed circulating antibodies and primed T cells and B cells (within hours to days).

7.2. Transplant rejection

Warfvinge et al. discuss how the immune rejection of a transplanted organ can be divided into three stages, where each stage starts if the previous stage is not activated or is adequately treated: Hyperacute rejection (HAR), delayed hyperacute rejection (DHR), and acute rejection (AR) (Warfvinge et al., 2006). HAR occurs in minutes to hours after implantation and is mediated by preformed antibodies against foreign antigens and directed mainly towards solid vascularized organs. HAR is not expected in subretinal transplants because of the nonvascularized nature of the subretinal space (Warfvinge et al., 2006). In addition, subretinal implants are not subject to the reperfusion damage encountered in many organ transplants (Loewendorf and Csete, 2013). DHR occurs within days if the graft does not induce HAR. DHR is also mainly antibody-mediated, with T-cells playing only minor roles. Lack of donor-specific vascular endothelium and antigen-presenting cells in RPE grafts make it unlikely that DHR is a component of RPE transplant rejection (Warfvinge et al., 2006). AR, mediated by CD4+ and CD8+ lymphocytes, occurs over days and weeks after the transplantation and is the main mechanism of rejection in allografts including allogeneic stem cell-derived cell replacement therapies. In a study examining the transplantation of mouse retinal progenitor cells into the pig subretinal space, xenografts were rejected after 3—5 weeks through a T-cell mediated process. Although anti-mouse antibodies were detected in pig serum, the authors concluded that a serum-mediated reaction was not the player in xenograft rejection (Warfvinge et al., 2006). Delayed chronic rejection, not mentioned earlier, may occur months after the transplantation and

is mediated through a combination of T-cell reactivation and antibodies formed against the donor cells.

7.3. Subretinal immune environment

While all of the above components contribute to immune recognition and rejection of transplanted allogeneic cells, a clear picture of the rejection hierarchy in the eye remains to be achieved. The eye has a unique advantage for a cell-based therapy from both immunological and clinical stand points. It is well established that the eye is an immune-privileged site, although the biological components of this property and their modulation under disease conditions are not fully understood as stated before.

RPE possesses characteristics that promote its survival even when transplanted to a non-immune-privileged site (Wenkel and Streilein, 2000). Thus, subretinal microenvironment immune privilege seems to be dependent on having an intact RPE monolayer (Wenkel and Streilein, 1998). Wenkel and Streilein grafted allogeneic fetal mouse RPE cells and RPE cells from CD95-deficient mice into an immune privileged site, the anterior chamber of the eye and into a non-immune privileged site, under the kidney capsule. Allogeneic fetal RPE grafts under the kidney capsule showed no immune rejection while CD95-deficient RPE cells promoted immune reaction leading to the rejection (Wenkel and Streilein, 2000). This may indicate that lack of apparent cell-mediated rejection of retinal tissue implanted in the subretinal space likely derives, at least in part, from the immune privilege capacity of RPE cell that protect them from immune rejection (Streilein et al., 2002). However, despite many similarities between hESC-RPE and endogenous, healthy RPE, the full capacity of hESC-RPE cells to regulate subretinal and retinal immune environment needs more work.

Radtke et al. grafted fetal retina/RPE tissue under the retina of patients with retinitis pigmentosa and advanced AMD (Radtke et al., 2008), observing no significant immune rejection reaction in their patients. The authors proposed that the absence of detectable graft damage, even in patients with donor-specific antibodies before implantation, would indicate that the blood-retina barrier is restricting antibody access into the subretinal space. In addition, they suggested that the subretinal space may induce an Anterior Chamber—Associated Immune Deviation (ACAID)-like process that would protect allogeneic cells placed in the subretinal space (Radtke et al., 2008) (Streilein et al., 2002). Others have proposed that the absence of immune rejection against the implanted allogeneic cells in the sub-retinal space depends on multiple other mechanisms including active processes involving CD95L (FasL) (Wenkel, 2000), programmed cell death ligand-1 (Sugita et al., 2009a, 2009b), and regulatory T cell differentiation through TGF-β secretion (Hirsch et al., 2015; Zamiri et al., 2005) and interleukin-10 (IL-10) secretion (Enzmann et al., 1998) as well.

Although intraocular spaces are, to a large extent, immune-privileged sites (Streilein et al., 2002; Zamiri et al., 2006; Zamiri et al., 2007), surgical trauma during the implantation of such cell therapies compromises the blood-ocular barrier and subjects surrounding cells to an increased level of recognition and reaction. Moreover, a major contributor to the pathogenesis of AMD is complement system dysregulation; thus, promoting cell survival in this pro-inflammatory microenvironment is a challenge for the long term success of SC-RPE replacement therapy.

While the role of the RPE in maintaining subretinal immunosuppressive status has been studied extensively, antigen presentation and alloreactive T cell differentiation are still not well understood in the eye. Evidence suggests that innate and adaptive components of the immune system are regulated through surface expression of molecules on RPE cells, as well as through autocrine

and paracrine effects of cytokines and growth factors secreted from the basal and apical sides of the cells. The RPE maintains an immunosuppressive role by suppressing both the adaptive and innate immune systems (Sugita et al., 2006; Sugita et al., 2008; Sugita et al., 2009a, 2009b; Horie et al., 2010; Sugita et al., 2010). Suppression of the adaptive immune system is mainly accomplished through secretion of TGF-β and thrombospondin, while the suppression of the innate immune system is primarily carried out by production of pigment epithelium-derived growth factor (PEDF) and somatostatin, which leads to the regulation of macrophage activation in subRPE and subretinal areas (Zamiri et al., 2005; Zamiri et al., 2006). In addition, the RPE induces macrophages to produce IL-10 which, in turn, down-regulates IL-12 production through autocrine signaling. LPS-stimulated macrophages produce higher levels of IL-10 and lower levels of IL-12 when cultures are supplemented with supernatants from murine RPE (Zamiri et al., 2005; Zamiri et al., 2006). IL-10 also stimulates production of TGF-β. TGF-β is a well-known anti-inflammatory cytokine that suppresses the major inflammatory transcription factor NFkB in macrophages and CD4+ lymphocytes, and promotes cytotoxic CD8+ and regulatory T cell activation (Akdis and Blaser, 2001; Levings et al., 2002; Zamiri et al., 2006). IL-10 is also known to down-regulate surface expression of MHC II and related co-stimulatory molecules in dendritic cells (Chang et al., 1994). This effect is, at least in part, due to the secretion of PEDF and somatostatin from RPE cells (Zamiri et al., 2006). On the other hand, there is some evidence showing that RPE cells can process and present antigens to T cells, stimulating their activation (Osusky et al., 1997). Lastly, in the anterior chamber of the eye, an antibody-mediated intraocular defense system (specifically antibody-dependent cell cytotoxicity [ADCC], which is moderated through complement-mediated chemotaxis of platelets, NK cells, and macrophages) is more active, although its role in subretinal immune reactions is not fully known.

The maturation status of RPE cells has effects on the immunosuppressive potential of these cells as polarized RPE is functionally different from nonpolarized RPE. Comparison of polarized hESC-RPE and fetal RPE shows comparable production of TGF-β2 from apical and basal compartments of both cell types (Hirsch et al., 2015). However, attainment of polarity is associated with a significant shift in growth factor and other cytokine production capacity of the cells (Sonoda et al., 2010). Whether hESC-RPE cells in suspension culture differ from sheets of attached, polarized hESC-RPE in secretion of immunomodulatory cytokines, inducing T cell apoptosis by Fas ligand expression, and inducing regulatory T cell differentiation should be clarified.

Regulatory T cells (Tregs) are a subset of CD4+ T cells that primarily regulate immune homoestasis by protecting tissues from aberrant immune reactivation (Sakaguchi et al., 2008) and function as professional tolerizing T cells with suppressive effects on effector T cells. Tregs are classified based on the surface molecules to 1) natural Tregs that express FoxP3, 2) inducible Treg, and 3) FoxP3 negative-IL-10-producing Tregs known as Tr1 cells (Hippen et al., 2011). Adoptive transfer of Tregs from has been proven to prevent immune rejection in graft-versus-host disease models (Hippen et al., 2011). In the eye, Tregs are part of numerous intraocular immunosuppressive mechanisms known as immune-privilege (Horie et al., 2010). Eye-specific Treg differentiation depends on TGF-β signaling. hESC-RPE monolayers have been shown to secret TGF-β2 similar to human RPE monolayers (Hirsch et al., 2015), suggesting their capability to convert naive T cells into regulatory T cells. The Treg population, in turn, mitigates T-cell proliferation, and inhibits interferon-gamma-induced expression of major histocompatibility complex class II on human RPE cells (Sugita et al., 2009a, 2009b). It should be mentioned here that, given the critical role of the Treg population in controlling intraocular inflammation, immunosuppressive therapies following transplantation surgeries should make sure not to target the Treg population (Loewendorf and Csete, 2013).

The occurrence of chronic loss of transplanted RPE cells in the subretinal space indicates that the immune privilege in the subretinal space is not perfect despite all these immunosuppressive mechanisms (Streilein et al., 2002). Zhang and Bok injected allogeneic RPE under the retina of RCS rats with and without pre-sensitizing them to the donor's antigens. In all experiments, subretinal grafts that were MHC I or MHC I and II incompatible showed progressive functional deterioration and loss (Zhang and Bok, 1998). In addition, they showed that MHC II can be expressed on the grafted RPE cells under appropriate conditions and promote functional decline of the transplanted cells (Zhang and Bok, 1998). Even if the subretinal space is an immune-privileged environment with conditions favorable for the implantation of allogeneic cells, its immunosuppressive condition may need reinforcement with perioperative immunomodulatory agents to provide the maximum protection for the limited number of cells introduced (Del Priore et al., 2003).

7.4. Immune response to stem cell-derived RPE and RPC

Depending on the source of stem cells, immune rejection can also be a major concern in stem cell-based therapies (English and Wood, 2011; Tang and Drukker, 2011). More discussion about the immune properties of hESC-RPE, iPSC-RPE, and RPC will follow; but it needs mentioning here that mesenchymal stem cells (MSCs) that are being tested for the treatment of retinal degeneration (Table 4), have the ability to actively suppress the immune system by inducing Tregs, suppressing B-cell and T-cell effector responses, and interfering with dendritic cell maturation (Griffin et al., 2010). Based on these properties, a range of MSC therapies have been proposed for degenerative, immunologic, and autoimmune diseases, including those of the eye (Joe and Gregory-Evans, 2010). Most current MSC-based clinical trials in the eye use autologous cells, making immune rejection not a major concern.

To determine if hESC-RPE could stimulate allogeneic immune responses, we exposed hESC-RPE to peripheral blood mononuclear cells (PBMC) from patients with AMD in a mixed lymphocyte reaction assay (MLR) (Okamura et al., 2007). MLR explores the capacity of allogeneic hESC-RPE cells to stimulate PBMC and is a used as an indicator for the ability of allogeneic cell to initiate cellular immune response (Fig. 4A). As shown in Fig. 4, hESC-RPE did not stimulate proliferation of peripheral blood monocyte cell (PBMC) as detected by BrdU assay (Fig. 4B). In addition, as complement- and antibody-mediated cell cytotoxicity is proposed as an innate immunity defense mechanism that can impose non-specific cytotoxic effects on allogeneic cells, we tested the ability of serum proteins for potential enhanced cell lysis of allogeneic hESC-RPE (Hewitt et al., 2007) (Fig. 4A). In our experiments, no difference in cell cytotoxicity was observed with exposure to active and heat-inactivated serum samples (Fig. 4C).

Although allogeneic hESC-derived transplants are subject to immune recognition and rejection, it has been shown that RPC derived from hESC bears lower immunogenicity compared to when it is derived from fetal retinal progenitors (Luo et al., 2014).

The third major type of stem cell-derived transplant, iPSC-derived cells, is theoretically identical to the patient's antigenic content and thus these cells are not supposed to induce an immune reaction. However, a publication by Zhao et al. has raised concerns about the possibility of post-translational modifications in iPSCs during their dedifferentiation/differentiation process, leading to slightly different antigenic properties of iPSCs (Zhao et al., 2011).

Table 4
Ongoing clinical trials for the use of stem cell-related cell therapies in retinal diseases.

Identifier ID	Study title	Date of registration (DD/mm/YYYY)	Primary sponsor	Locations/Collaborators	Estimate enrollment	Cell source	Target disease	Estimated completion date	Delivery methods	Phase
NCT01344993	Safety and Tolerability of Sub-retinal Transplantation of hESC Derived RPE (MA09-hRPE) Cells in Patients With Advanced Dry Age Related Macular Degeneration (Dry AMD)	28-04-2011	Ocata Therapeutics, Inc.	Jules Stein Eye Institute, California; Bascom Palmer Eye Institute, Florida; Mass Eye and Ear, Massachusetts; Wills Eye Institute, Pennsylvania	16	hESC-RPE	Dry AMD	Dec-2014	Subretinal cell suspension injection	Phase I/II
JPRN-UMIN000011929	A Study of transplantation of autologous induced pluripotent stem cell (iPSC) derived retinal pigment epithelium cell sheet in subjects with exudative age related macular degeneration	10-02-2013	RIKEN Institute	RIKEN, the IBRI Hospital and the Kobe City Medical Center General Hospital, Japan	6	iPSC-RPE Cell Sheet	Exudative AMD	4 years after transplantation	Subretinal cell sheet implantation	N/A
Not assigned yet	Phase 1/2a Safety Study of Subretinal Implantation of CPCB-RPE1 (Human Embryonic Stem Cell-Derived Retinal Pigment Epithelial (RPE) Cells Seeded on a Polymeric Substrate) in Subjects with Advanced, Dry Age-Related Macular Degeneration (AMD).	n/a	Regenerative Patch Technologies (RPT)	University of Southern California, University of California, Santa Barbara	6–10	hESC-RPE	Dry AMD (GA)	2016	Subretinal cell sheet (on a parylene membrane) implantation	Phase I/II
NCT01632527	Study of Human Central Nervous System Stem Cells (HuCNS-SC) in Age-Related Macular Degeneration (AMD)	21-06-2012	StemCells, Inc	Retina-vitreaous Associates Medical Group and Byers Eye Institute at Stanford, California; New York Eye and Ear Infirmary, New York; Retina Research Institute of Texas and Retina Foundation of the Southwest, Texas	15	HuCNS-SC	Dry AMD	Jun-2015	Subretinal cell suspension injection	Phase I/II
NCT02286089	Safety and Efficacy Study of OpRegen for Treatment of Advanced Dry-Form Age-Related Macular Degeneration	02-11-2014	Cell Cure Neurosciences Ltd.	Hadassah Ein Kerem University Hospital, Israel — Not yet recruiting	15	hESC-RPE	Dry AMD	Aug-2017	Subretinal cell suspension injection	Phase I/II
NCT01691261	A Study Of Implantation Of Human Embryonic Stem Cell Derived Retinal Pigment Epithelium In Subjects With Acute Wet Age Related Macular Degeneration And Recent Rapid Vision Decline	19-09-2012	Pfizer	University College, London, United Kingdom	10	hESC-RPE	Wet AMD	Jun- 2017	Subretinal implantation of monolayer cells on a polyester scaffold	Phase I

(*continued on next page*)

Table 4 (*continued*)

| Identifier ID	Study title	Date of registration (DD/mm/YYYY)	Primary sponsor	Locations/Collaborators	Estimate enrollment	Cell source	Target disease	Estimated completion date	Delivery methods	Phase
NCT01736059	Clinical Trial of Autologous Intravitreal Bone-marrow CD34 + Stem Cells for Retinopathy	25-07-2012	University of California, Davis	University of California, Davis, California	15	BMSC	Dry AMD, DR, RVO, RP, Hereditary Macular Degeneration	Dec-2014	Intravitreal injection	Phase I
NCT01226628	A Safety Study of CNTO 2476 in Patients With Age-Related Macular Degeneration	21-10-2010	Janssen Research & Development, LLC	Arcadia, California; Philadelphia, Pennsylvania	24	hUTC	Dry AMD	Mar-2015	Subretinal cell suspension injection	Phase I
NCT01674829	A Phase I/IIa, Open-Label, Single-Center, Prospective Study to Determine the Safety and Tolerability of Sub-retinal Transplantation of Human Embryonic Stem Cell Derived Retinal Pigmented Epithelial(MA09-hRPE) Cells in Patients With Advanced Dry Age-related Macular Degeneration(AMD)	22-08-2012	CHA Bio & Diostech	CHA Bundang Medical Center, Korea	12	hESC-RPE	Dry AMD	Apr-2016	Subretinal cell suspension injection	Phase I/II
NCT01920867	Stem Cell Ophthalmology Treatment Study (SCOTS)	08-08-2013	Retina Associates of South Florida & MD Stem Cells	Retina Associates of South Florida, Florida; Al Zahra Private Hospital, United Arab Emirates	300	hBMSC	AMD, Hereditary Retinal Dystrophy, Optic Nerve Disease, Glaucoma	Aug-2017	Intravitreal and subretinal cell suspension injection	N/A
NCT01518127	Intravitreal Bone Marrow-Derived Stem Cells in Patients With Macular Degeneration	20-09-2011	University of Sao Paulo	Rubens Siqueira Research Center, Brazil	10	Autologous BMSC	AMD	Dec-2015	Intravitreal injection	Phase I/II
CTRI/2010/091/00639	A Clinical Trial to Evaluate the Effect of Bone Marrow Derived Stem Cell in Diseases Like Dry Age Related Macular Degeneration and Retinitis Pigmentosa	07-05-2010	Indian Council Of Medical Research, India	All India Institute of Medical Sciences, Ansari Naga, India	50	BMSC	dry AMD, RP	April 2010 to 3 year (still Open to Recruitment)	Intravitreal injection	Phase I
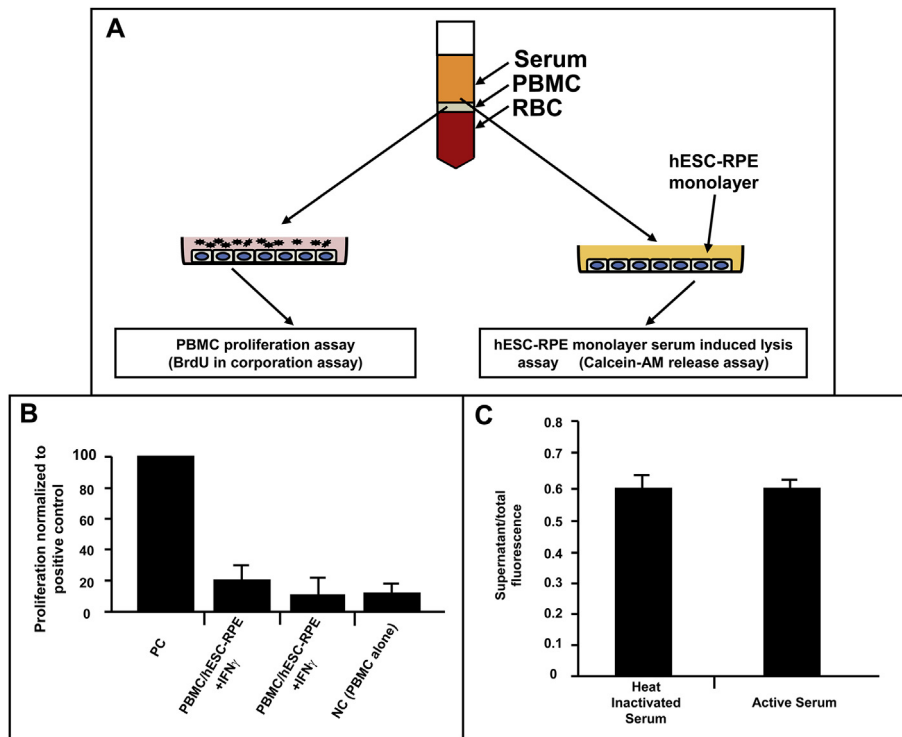
Fig. 4. Testing human embryonic stem cell-derived retinal pigmented epithelium (hESC-RPE) ability to stimulate allogeneic immune responses. (A) Schematic drawing of the steps involved in mixed lymphocyte reaction (MLR) and serum lysis assays. The MLR assay uses BrdU incorporation to investigate whether allogeneic hESC-RPE cells can stimulate proliferation of peripheral blood mononuclear cells (PBMC) from a patient blood sample. The serum lysis assay uses Calcein-AM release measurements to investigate whether serum proteins from a patient blood sample can enhance cell lysis of allogeneic hESC-RPE. (B) When performing MLR assays using PBMCs isolated from patients with age-related macular degeneration (AMD), hESC-RPE was found to not stimulate the proliferation of PBMC. Pre-stimulation of hESC-RPE with interferon gamma (IFNγ) did not increase PBMC proliferation. (NC: negative control; PC: positive control). (C) When performing serum lysis assays using peripheral serum from a patient with AMD, no difference in cell cytoxicity of hESC-RPE was observed when comparing exposure to active and heat-inactivated peripheral serum. (Error bars indicate standard error of the mean [SEM]).

These differences may make autologous iPSC-derived cells subject to immune reaction. That said, subsequent studies assessing immunogenicities of multiple iPSC lines have demonstrated only negligible immune response to iPSC-derived cells after transplantation (Tan et al., 2014).

As stated before, it is known that the subretinal immunosuppressive microenvironment provides a favorable shelter for transplanted allogeneic hESC-RPE and RPC from immune recognition. Multiple studies have shown that hESC-RPE cells injected into the subretinal space survive short term without evident immune inflammation (Diniz et al., 2013; Hambright et al., 2012; Lu et al., 2009). However, even in the absence of acute rejection, long term (over months and years) viability and function of the implanted cells in the presence of potential low grade of inflammation has not been clarified. Better understanding of the process leading to the loss of these cells is critical for the clinical translation of stem cell-derived RPE and RPC replacement therapies.

7.5. Strategies to prevent immune rejection of stem cell-derived RPE implants

While the immunopriviledged subretinal microenvironment may provide some protection against an inflammatory response to stem cell-derived RPE implantation, even slight immune infiltration may be irreversibly deleterious to the intimate relationship between photoreceptors and the limited number of transplanted RPE cells (about 50,000—200,000 cells). Careful patient selection and a robust immunosuppressive regimen consisting of pre- and post-operative use of immunomodulatory therapy (mycofenolate mofetil and tacrolimus), plus oral and topical corticosteroids, has

been practiced in the current clinical trials so far (Schwartz et al., 2012, 2015). But, even with a robust anti-inflammatory regimen, gradual loss of allogeneic RPE and xeno-RPE starts 1—2 month after implantation in the subretinal space in animal models (Stanzel et al., 2014; Crafoord et al., 2000). Whether intraocular depot corticosteroid injections, such as intravitreal dexamethasone and fluocinolone acetonide implants can help decrease the chance of immune reaction to the transplanted cells or not is yet to be determined (Ahmad et al., 2012; Tomkins-Netzer et al., 2014). Below, we discuss the additional factors that should be considered in stem cell-derived RPE transplantations to minimize chances of inflammatory response.

Local inflammation seen after subretinal implantation can be induced by a number of factors, including allogenic moieties on the transplanted cells, inflammation associated with surgical procedures, and any artificial scaffold to which cells are attached, such as in the case of implanting hESC-RPE sheets attached to an underlying support. Control of allogeneic immune responses by the host is an obvious area where the inflammatory response can be controlled. Although it is almost impossible to find a perfectly matched donor and recipient (except between identical twins), matching MHC, ABO, and minor histocompatibility antigens as much as possible improves graft survival significantly. Allofactors other than MHC antigens are increasingly recognized as a heterogenous group of targets for immune recognition of transplanted cells. The importance of such molecules is stressed by observation of transplant rejection in HLA-identical siblings (Grafft et al., 2010). As Thomson et al. suggested in their original publication, generation of a pool of immunologically diverse stem cell-derived RPE cells with known MHC genotypes may ensure that transplant

candidates have access to RPE cells with low mismatch to their own cells (Thomson et al., 1998). To determine the feasibility of creating such hESC-RPE or iPSC-RPE banks, it is necessary to have a rough estimate of how many different RPE cell lines would need to be in such a pool to have the maximum number of possible combinations of factors to match to donors as has been reported by Taylor et al. and Nakajima et al. (Taylor et al., 2005; Nakajima et al., 2007).

Intraocular surgery by itself, including vitrectomy and cataract surgery, has been associated with intraocular inflammation as indicated by clinical findings such as post-operative cystoid macular edema, epiretinal membrane formation and vitreous or aqueous cell and proliferative vitreoretinopathy. In most cases, it is not possible to separate inflammation that is due to a primary pathological process (e.g. vein occlusion, uveitis, retinal detachment etc.) from the actual surgical procedure. However, large retrospective studies performed on subjects who have undergone pars plana vitrectomy only for symptomatic floaters (with no other ocular comorbidity) show a ~5—10% rate of post-operative findings such as cystoid macular edema, epiretinal membrane formation and hypotony which are suggestive of some degree of surgically induced intraocular inflammation (de Nie et al., 2013). In addition, some studies suggest that multiple intraocular surgeries predispose to increased inflammation as measured by the presence of cystoid macular edema after the second surgery (Mylonas et al., 2013). Perhaps most importantly, studies show that cystoid macular edema occurs in up to 28% of eyes after pars plana vitrectomy for retained lens fragments (Merani et al., 2007) and in 46% of patients with aphakia or anterior chamber intraocular lenses (Cohen, 2006). This suggests that patients selected for stem cell therapy should optimally receive some form of peri-operative immunosuppression, have no history of ocular surgery and every attempt should be made to keep such patients bicameral (i.e phakic or pseudophakic). Ideally, these patients should undergo cataract surgery at the same time or before vitrectomy procedures and there should be a zero tolerance for residual lens components that may serve as a source of chronic inflammatory stimuli. It should also be mentioned here that minimizing the surgical manipulation by injecting cell suspension rather than implanting RPE sheet through a retinotomy (that is inherently a more traumatic procedure) should be weighed against the benefits of implanting polarized stem cell-derived RPE sheets on an artificial platform. Whether the potential risk imposed by surgical trauma for implantation of hESC-RPE compromises the implant survival and function will be addressed in trials that use such implants.

As alluded to above, the scaffold upon which monolayers of stem cell-derived RPE cells are grown and delivered into the subretinal space is another source of potential inflammation. Immunologically inert platforms for hESC-RPE sheets, such as parylene membrane, that is proposed in the California Institute for Regenerative Medicine (CIRM)-sponsored California Project to Cure Blindness trial at the University of Southern California, is a biocompatible material extensively used in medical biodevices and has not been associated with any immune reactivity (Hu et al., 2012; Lu et al., 2012; Lu et al., 2014). While many other non-biodegradable and biodegradable materials are being tested as scaffolds for stem cell-derived RPE monolayers (section 11.3), an alternative is to inject a stem cell-derived RPE suspension instead of transplanting cells on a scaffold. As mentioned before, cell suspension injection confers minimal surgical trauma and does not predispose to an immune reaction that may be mounted against an artificial platform. However, there are major concerns about the ability of suspended cells to become a polarized monolayer in the subretinal space (Schwartz et al., 2015) and RPE cells in suspension show lower tolerance to oxidative stress than those in monolayers (Hsiung, 2015). The answer may lay in products similar to the RIKEN's iPSC-RPE sheets that are grown on gelatin where this gelatin layer is dissolved enzymatically before the implantation; thus, providing an RPE monolayer implant without a potentially immunogenic or functionally disturbing scaffold. Of course, manipulating such monolayers that are not grown on a platform (e.g., obtaining proper orientation in the subretinal space) would add another challenge to the implantation procedure.

7.6. Future approaches to address immune reaction for implanted hESC-RPE

Despite the immune-privileged status of the subretinal space, histoincompatibility and immune rejection remains a major challenge for RPE transplantations that necessitates both systemic and local immune suppression. This is partly because the surgical procedure to transplant hESC-RPE cells compromises the blood-ocular barrier and exposes the transplanted cells to the host's immune system. Given the high morbidity associated with systemic immune suppression, particularly in the aged population characteristic of AMD patients, modulation of primarily the local immune system to down-regulate the immune reaction against transplanted cells will enhance the long-term survival and success of hESC-RPE transplantation. A potential way to reduce the immunogenicity of allogeneic cells intended for use in cell replacement therapies is to reduce the expression of MHC-II antigens on cell surface.

MHC-II role in immune reaction to hESC-RPE: As stated earlier, the process of graft rejection begins with the recognition of allogeneic antigens on the surface of the transplanted cells (Kohen et al., 1997). MHC antigens play a central role in immunologic rejection of neural grafts. MHC-II antigens on graft cells may be recognized as exogenous antigens or they may contribute to the presentation of antigens to the host immune cells (Sloan et al., 1991). MHC-I antigens are constitutively expressed in all nucleated cells, including RPE cells (Fig. 5A) (Zhang and Bok, 1998). MHC-II expression is naturally limited to antigen-presenting cells, but it can be expressed on nonlymphoid cells in certain circumstances and may render them capable of effectively presenting organ-specific antigens to T-cells and, thus, initiate an immune response (Feldmann et al., 1987; Casella et al., 1999). For example, RPE cells can express MHC-II on their surface upon stimulation by pro-inflammatory cytokines, such as interferon-gamma (Percopo et al., 1990; Casella et al., 1999). MHC-II expression on RPE is notable because RPE cells expressing MHC-II are capable of presenting their antigens to CD4+ helper T-cells (Sun et al., 2003; Gregerson et al., 2007). In turn, helper T-cells can coordinate an immune response that may include recruitment of phagocytes and/or production of antibodies via B-cell activation (Sun et al., 2003). This process has been proposed to be the primary one involved in the rejection of RPE transplants (Kohen et al., 1997; Enzmann et al., 1999). Down-regulation of MHC-II on grafted tissues is thought to prevent graft rejection (Yang and Liu, 2008). It has also been specifically shown that suppression of MHC-II expression on transplanted RPE cells is essential for long-term survival (Kohen et al., 1997; Enzmann et al., 1999). Major proof for the critical role of MHC-II expression in immune recognition of allogeneic cells comes from placental trophoblastic cells; the lack of MHC-II antigens presented on these cells is considered to be the main reason these cells escape maternal immune recognition (Trempe, 2011). Further evidence of the importance of MHC-II expression on the immunogenicity of stem cells is derived from experiments showing that the higher immunocompatibility of mesenchymal stem cells is partly because of the lack of expression of MHC-II and costimulatory molecules on these cells (Feldmann et al., 1987; Allikmets et al., 2009). Therefore, down-regulation of MHC-II expression is proposed as a novel therapeutic tool to improve survival of the transplants (Wright and Ting, 2006).
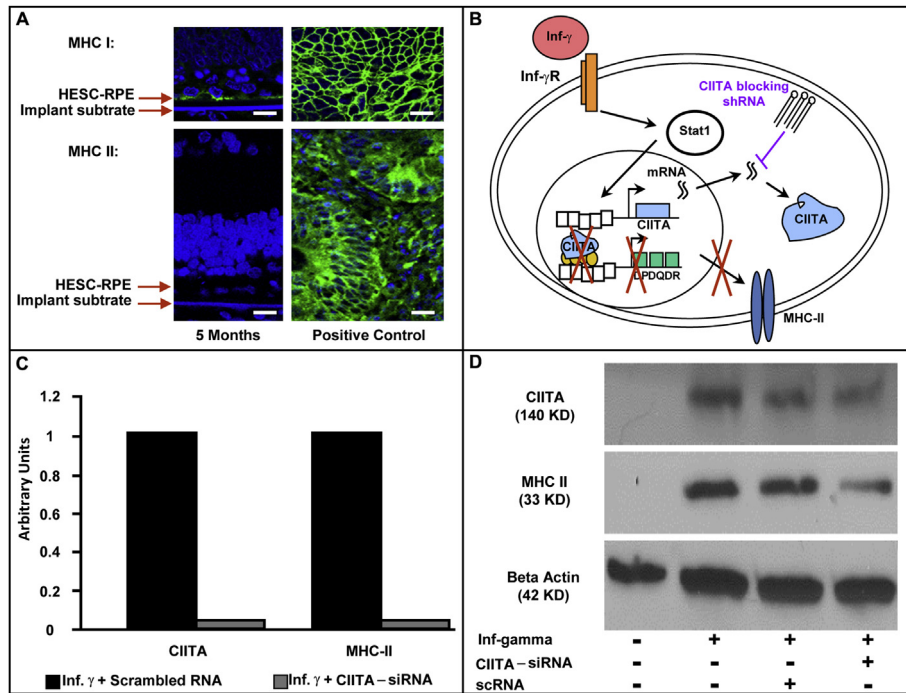
Fig. 5. Knock down of MHC-II expression using *CIITA*-siRNAs. (A) MHC-I and MHC-II expression on hESC-RPE implanted in subretinal space of RCS rats (scale bars: 20 μm). (B) Proposed pathway depicting how shRNA/siRNA transfection to silence *CIITA* may result in down regulation of MHC-II expression. (C) Fold change in CIITA and MHC-II mRNA expression after interferon gamma stimulation of anti-CIITA siRNA transfected hESC-RPE. (D) Western blot analysis confirming knock down of MHC-II expression.

MHC-II expression is regulated at both translational and post-translational levels. Epigenetic regulation of MHC-II gene expression includes MHC-II transactivator (CIITA) and regulatory factor X, two proteins that serve as focal points for recruiting histone-modifying enzymes to MHC-II promotors (Folk and Stone, 2010; Trempe, 2011) (Fig. 5B). An established approach to down-regulating gene expression uses small interfering RNA (siRNA) and short hairpin RNA (shRNA), which is a sequence of RNA that makes a tight hairpin turn that silences target gene expression via RNA interference (RNAi) (Fig. 5B). Although major safety concerns overshadow the use of shRNAs as therapeutics, advancements in molecular biology have allowed for improved design of the siRNA/shRNA molecules (Burnett et al., 2011). For example, shRNA silencing of immunosuppressive cytokine genes is being developed to specifically enhance immune reaction to advanced cancers (Fine et al., 2000).

Using shRNA to silence the CIITA gene could induce a long-lasting silencing of MHC-II expression on hESC-RPE thus limiting their recognition by the immune system. We have shown that upon transfection of siRNA to silence *CIITA*, interferon gamma-induced expression of the HLA-DR gene can be knocked down by about 95% (Fig. 5C and D). Optimizing a similar approach to ex-vivo reduction of MHC-II expression on hESC-RPE transplants may improve hESC-RPE survival upon transplantation. Although sub-optimal transfection efficacy could leave a subset of cells that may eventually express MHC-II and induce an immune response to the hESC-RPE transplant, the immune reaction would be less robust, possibly reducing the need for strong and long-term immunosuppression.

8. Pre-clinical animal studies for cell replacement therapies for AMD

8.1. AMD animal models − RPE degeneration

Despite the anatomic and functional differences of the retina,

RPE, and choroid structure between rodent and human eyes, rodent models have been extensively used to define the pathogenesis of AMD and test treatments. One of the key differences between the rodent (specifically mouse and rat) and human retina is that the former does not have a fovea and its photoreceptors are mainly composed of rods. There are additional differences between murine and primate eyes in RPE, Bruch's membrane structure, and retinal organization that probably reflect differences in retinal signal processing (Jeon et al., 1998; Ramkumar et al., 2010). In addition, no animal model fully represents the pathologic changes seen in human AMD. Nevertheless, rodent models have been instrumental in the development of ocular therapeutics, including cell-based regenerative treatments.

The choice of the animal model depends on the kind of therapy being studied, with more than one model being potentially necessary (Pennesi et al., 2012; Bharti et al., 2014). In general, there are three main groups of genetically-modified rodents: (1) genetically-engineered murine models with alterations in genes related to juvenile macular dystrophies or AMD (including inflammatory genes, oxidative stress-associated genes, metabolic pathway genes, and lipid metabolism genes); (2) immunologically-manipulated mice; and (3) naturally occurring mice with accelerated senescence that develop photoreceptor atrophy and thickening of Bruch's membrane (similar to the AMD phenotype) (Ramkumar et al., 2010). Below we summarize the small and large animal models that are currently used in stem cell research for AMD.

8.1.1. Royal College of Surgeons rats

The Royal College of Surgeons (RCS) rat is the animal model most commonly used for RPE-related diseases, including AMD. RCS rats suffer from a mutation in the c-mer proto-oncogene tyrosine kinase (*MERTK*) gene that leads to disruption of outer segment phagocytosis by the RPE and retinal degeneration (D'Cruz et al., 2000). *MERTK* codes for a protein essential for the phagocytosis of the photoreceptor outer segments. Extrapolating data from RCS rats

to humans is difficult, since the former has a much lower density of cones and no macula as mentioned above. Additionally, the Bruch's membrane of the RCS rat is young and thin when it is under study, which differs from the aged, pathologic Bruch's membrane of an AMD patient (Ramsden et al., 2013). Despite its limitations, the RCS rat is an FDA-accepted model for studies on stem cell-derived RPE transplantation for retinal degeneration. The dystrophic RCS rat is born with a retina that appears morphologically normal, but as early as postnatal day (P) 16, the outer nuclear layer begins to degenerate progressively and the animal loses vision. By P105, only a few scattered cone photoreceptors remain in the retina (Strauss et al., 1998; Blenkinsop et al., 2012; Ramsden et al., 2013). Late replacement of the degenerated RPE cells with subretinal injection of the human RPE cell line cells, ARPE-19, in P60 RCS rats has been shown to rescue the degenerating photoreceptors, although the results were not as impressive as when RPE transplantations were performed on P21 (Wang et al., 2008). hESC-RPE that underwent subretinal implantation in RCS rats survived in the rats for >200 days with improvements in retinal function (Lund et al., 2006) and without any signs of uncontrolled cell proliferation or teratoma or tumor formation (Lu et al., 2009).

Subretinal transplantation of RPE cells derived from both iPSC and hESC into the RCS rat has been able to rescue declining vision to varying degrees (Li and Turner, 1991; Little et al., 1996; Sauve et al., 2002; Arnhold et al., 2006; Pinilla et al., 2007; Wang et al., 2008; Park et al., 2011; Diniz et al., 2013). Whether this rescue of vision is due to replacing the lost RPE cells or the paracrine effects of growth factors secreted by the implanted RPE cells remains to be determined. It is certainly possible that a combination of both mechanisms is responsible for rescue of photoreceptors.

8.1.2. Nonhuman primate models

The retina of nonhuman primates, like the human retina, has a macula and recognizable early and intermediate stages of AMD occur naturally in some species. Macular drusen appear in 50% of a specific colony of Rhesus Monkey (*Macaca mulatta*) older than 9 years of age and almost all of them when they are 25 and older (Zeiss, 2010). Advanced AMD and geographic atrophy, however, are rarely part of the natural history of the macular degenerative disease in these animals, which limits their use as an AMD model. However, nonhuman primates are being used in cell replacement therapies. Immune rejection of allogeneic iPSC-RPE transplants is studied by subretinal transplantation of iPSC-RPE derived from cynomolgus monkey (*Macaca fascicularis*) by Takahashi's group in Japan (Kamao et al., 2014).

8.1.3. Surgical models for safety studies

Although almost all stem cel-derived RPE efficacy and safety studies have been performed in small animals, surgical techniques and tools must be developed and tested in eyes with a size comparable to the human eye before such treatments can move to human trials. In other words, larger eyes are required when larger human size implants are studied for assessing safety and surgical feasibility of transplantation. Thus, rabbits, dogs, monkeys, and pigs (e.g. Yucatan minipigs), all having eye sizes close to the human's, have a possible role in pre-clinical studies on stem cell therapies (Bharti et al., 2014). Despite the limitations for experiments on larger animal eyes (e.g. logistics and expenses), the porcine model seems to be superior to other models because of similarities between porcine and human eyes that offer potential for direct clinical translation to human subjects (Klassen et al., 2007).

8.2. Transplantation approaches and pre-clinical outcomes

The eye is a particularly suitable organ for transplantation because of its relatively easy perioperative accessibility with non-invasive imaging methods. Minimally invasive surgical techniques for the transplantation procedures (e.g. pars plana vitrectomy) are well-developed. In addition, RPE, a critical tissue for vision and a specific target for replacement therapy, has a relatively simple histologic structure; It is a monolayer of uniform cells, without direct vascular supply or neural innervation, that sits over a thin pentalaminar structure (Bruch's membrane). RPE monolayer sheets, or RPE cell suspensions, can be fairly easily transplanted under the retina by creating holes or flaps in the retina, a technique that is already used in vitreoretinal surgery. Most importantly, the retina is an immune-privileged site, which provides an additional measure of protection to implanted cells.

8.2.1. Stem cell-derived RPE suspension injection

Transplantation of stem cell-derived RPE cells have evolved since the early studies using autologous RPE grafts (Majji and de Juan, 2000; Phillips et al., 2003; Joussen et al., 2006). Now, there are two main approaches to deliver cells into the subretinal space. First is the injection of a stem cell-derived RPE suspension into subretinal space and the second approach involves engrafting a monolayer of stem cell-derived RPE cells seeded on a supporting membrane. Safety and tolerability of subretinal injection of hESC-RPE cell suspension is currently being tested in a multicenter clinical trial (Schwartz et al., 2015). Cell suspensions may provide beneficial effects by integrating into the host PRs and functioning as indigenous RPE (Schwartz et al., 2012) and/or, by secreting growth factors and trophic molecules as well as scavenging debris, in the subretinal space (Bharti et al., 2011; Li et al., 2011).

In initial implants into monkeys, RPE cells were transplanted using the open–sky vitrectomy technique, which later evolved to the closed-eye methods described below (Gouras et al., 1985, 1984; Lopez et al., 1987). Currently, the delivery of a cell suspension is being performed by two different techniques. The first one is an internal approach and involves pars plana vitrectomy, removal of the posterior hyaloid, and creating a small retinotomy (Klimanskaya et al., 2004; Schwartz et al., 2012, 2015). The cells are then injected via the retinotomy, creating a localized retinal detachment or bleb (da Cruz et al., 2007). This technique has been tried in the eyes of humans and large animals as well as in rats, rabbits, and cats (Lund et al., 2001a, 2001b) for RPE transplantations with some modifications such as avoiding vitrectomy. Small animals present a significantly larger lens and a smaller vitreous cavity compared to their globe size, which makes this procedure considerably more difficult to perform on these animals (da Cruz et al., 2007).

The second cell suspension delivery approach reaches the subretinal space via an external path. A localized peritomy is followed by the dissection of the dorsal sclera and choroid, creating room for insertion of a needle, which injects the cells into the subretinal space. This technique does not require vitrectomy nor retinotomy. (da Cruz et al., 2007) and is the preferable method for use in small animals, such as mice (Jiang et al., 1994), rats, and rabbits (Wong-pichedchai, 1992), for the reasons described above. In our experience with rats (RCS and Athymic nude), after peritomy is performed, a 27-gauge needle is used to make the incision through the sclera and choroid, and also to perform an anterior chamber paracentesis. The latter lowers the intraocular pressure to reduce the efflux of subretinal fluid and, therefore, the outflow of injected cells. An injection of saline solution is performed prior to the cell suspension injection, creating a local retinal detachment. A 32-gauge blunt cannula, Hamilton syringe (Hamilton, Reno, NV), is used to inject the cell suspension (Lund et al., 2006; Lu et al., 2009; Diniz et al., 2013; Hu et al., 2012). In terms of the amount of cells injected in each eye, previous studies have described different

quantities that vary from 5×10^3 to 1×10^5 cells/eye in RCS rats (Lund et al., 2006; Lu et al., 2009; Duan et al., 2013). When the choroid is dissected 1–2 weeks after the injection, a small, localized hemorrhage is usually seen near the site of injection. In some cases, however, a larger amount of blood may be found subretinally, compromising the surgical outcome. Additionally, the rupture of Bruch's membrane may cause inflammation and lead to immune reactions not present in the internal approach (da Cruz et al., 2007).

Because RPE physiology is dependent on its structure as a polarized monolayer of cells adherent to each other by tight junctions, it is essential that stem cell-derived RPE cell suspensions ultimately form a monolayer in order to perform its normal functions such as phagocytosis of photoreceptor outer segments and to form the outer blood ocular barrier. An earlier study of subretinal injection of autologous rabbit RPE in suspension form showed that RPE cells can form a monolayer under the retina that can persist up to 6 months (Crafoord, 1999). But, in a more recent study, fluorescently-labeled iPSC-RPE cell suspensions injected into the subretinal space of monkeys showed that cells preferentially accumulated at the lower margin of the bleb 7 days post-injection, compromising monolayer formation. Reflux of injected cells into the vitreous is also a major problem given that these cells can be a potential trigger for proliferative vitreoretinopathy. In contrast, transplanted monkey iPSC-RPE sheets did not move after implantation in the subretinal space (Kamao et al., 2014). An additional concern for using the cell suspension transplant technique is the possibility of formation of multilayered clumps of cells that could damage the overlying retina.

It has been shown that aged Bruch's membrane does not adequately support RPE adhesion, survival, and function of transplanted RPE cells (Gullapalli et al., 2004; Gullapalli et al., 2005; Sun et al., 2007; Tezel et al., 2007; Tezel et al., 1999; Pan et al., 2013). Not only is the adhesion of RPE cells to Bruch's membrane from aged donors and AMD patients delayed (i.e. by up to 3 weeks), but using an aged Bruch's membrane also hinders the formation of functional monolayers (Sugino et al., 2011). Multiple methods to enhance RPE adhesion to Bruch's membrane are reviewed by Heller and Martin (Heller and Martin, 2014). Of these, manipulating RPE cells to overexpress integrin family molecules in order to improve the binding and proliferation of RPE cells on Bruch's membrane seems most promising (Fang et al., 2009; Afshari et al., 2010). However, until the challenge of RPE adhesion to Bruch's membrane and attainment of an RPE monolayer is overcome, subretinally injected suspensions of hESC-RPE cells will suffer from gradual loss and dysfunction of the implanted cells (Diniz et al., 2013).

8.2.2. Polarized stem cell-derived RPE cells

Aiming to reproduce the natural anatomy of the RPE as a polarized monolayer, strategies for transplanting stem cell-derived RPE using intact cell sheets or RPE cells grown on a Bruch's Membrane-like prosthesis have been broadly developed. A carrier substrate that mimics Bruch's membrane, either permanently or transiently, might serve as an anchor for the cells, promote polarization, and support a monolayer structure, while also allowing a better controlled surgical delivery into the subretinal space. Multiple studies, including several from our group, show that hESC-RPE cells in polarized monolayers more closely mimic the genuine human RPE layer compared to hESC-RPE suspensions. Hsiung et al. compared the survival of polarized, nonpolarized/confluent, and nonpolarized/subconfluent hESC-RPE cells following oxidative stress treatment with different doses of H_2O_2 (Hsiung et al., 2014). Apoptosis assays revealed the highest amount of cell death to be in subconfluent hESC-RPE cells, and little cell death in polarized hESC-RPE cells, following the oxidative stress treatment. For example, while most nonconfluent cells underwent cell death with 600 uM

H_2O_2 treatment, no dead cells were detected in treated polarized cells There were additionally higher levels of proapoptotic factors in the nonpolarized RPE cell groups relative to the group of polarized cells. The polarized RPE cells also had constitutively higher levels of cell survival and antiapoptotic signaling factors (such as p-Akt and Bcl-2), as well as antioxidants (specifically superoxide dismutase 1 and catalase), when compared to nonpolarized cells, possibly contributing to higher tolerance of polarized hESC-RPE to oxidative stress. The authors suggest that polarized hESC-RPE monolayers implanted in the subretinal space of patients with AMD should have an improved survival compared to hESC-RPE cells injected in suspension (Hsiung et al., 2014). TGF-β secreted from RPE cells is a major component of subretinal immunosuppressive status. On other hand, high levels of TGF-β may promote cell migration and fibrosis. Hirsch et al. compared TGF-β secretion of nonpolarized human RPE, polarized human RPE, and polarized hESC-RPE and reported no significant difference in production levels between polarized human RPE and polarized hESC-RPE. Additionally, the concentration of TGF-β2 was also significantly higher in supernatants from cultures of nonpolarized RPE cells (Hirsch et al., 2015). This may mean that moderated levels of TGF-β2 produced by polarized hESC-RPE, more similar to physiologic conditions, offers an immunosuppressive environment in the subretinal space. The authors also suggest that there is a higher risk of subretinal fibrosis with hESC-RPE cell suspension transplantation because of higher secretion of TGF-β from non-polarized RPE, although such fibrosis has not been reported as a major adverse event in the current hESC-RPE suspension transplantation trial (Schwartz et al., 2012).

Many groups have focused on culturing RPE cells on different substrates to be transplanted as a patch. Artificial substrates must be inert to prevent rejection and must allow diffusion of small and mid-range sized molecules. They additionally need to be flexible to facilitate delivery beneath the macula, but also strong enough to preserve the cells as a monolayer. Among several biomaterials, plasma polymers, Parylene C, and polyimide and polyester membranes are potential substrates under evaluation in preclinical research (Kearns et al., 2012; Subrizi et al., 2012; Diniz et al., 2013; Stanzel et al., 2014) (Carr et al., 2013; Ramsden et al., 2013).

The properties of Parylene C, such as its mechanical strength, biostability, and chemical inertness, make this substrate suitable for use in implantable devices. The submicron Parylene C membrane, developed by our group, is an ultrathin, 0.3 μm thick film, supported on a 6 μm thick mesh. It has been demonstrated that when Parylene thickness is reduced to 0.15–0.30 μm, this substrate possesses characteristics similar to those of the Bruch's membrane in humans, functioning as a semi-permeable membrane (Lu et al., 2012). Furthermore, in vivo studies have also shown positive outcomes; at one week after subretinal implantation of RCS rats and normal Copenhagen rats with polarized monolayers of hESC-RPE cells cultured on ultrathin Parylene C, retinal morphology was found to be preserved in histological sections and using SD-OCT. Also, RPE monolayer structure on the substrate was maintained with only minor cell loss (Hu et al., 2012).

Results from another in vivo study suggest that hESC-RPE cell survival is improved when cells are transplanted as a monolayer on Parylene substrates compared to injection as a cell suspension. Immunocompromised nude rats were used to investigate tumorigenicity and cell survival after subretinal transplantation of hESC-RPE cells, either as a cell suspension or monolayer on a Parylene membrane. Both approaches were demonstrated to be safe, with no tumor formation but, cell survival and RPE maintenance as a monolayer were significantly higher for hESC-RPE cells transplanted on a Parylene membrane compared to injection as a cell suspension (Diniz et al., 2013).
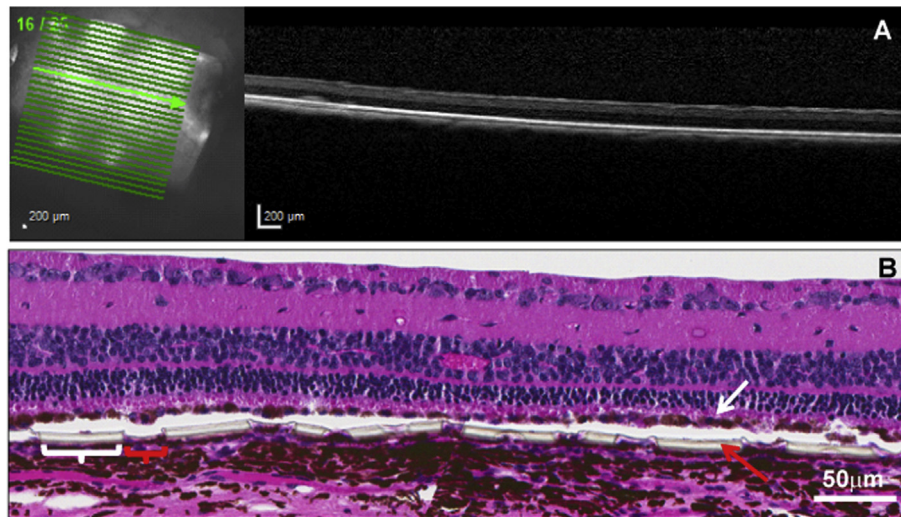
Fig. 6. Human embryonic stem cell-derived retinal pigment epithelium (hESC-RPE) sheets seeded on an ultrathin Parylene membrane transplanted in the subretinal area of porcine eye. (A) infrared image and optical coherence tomography scan showing the positioning of the hESC-RPE sheet seeded on a Parylene membrane under the retina. (B) hematoxyline and eosin stained retinal section reveals the subretinal positioning of the Parylene membrane. Note that pigmented hESC-RPE cells (white arrow) is artificially detached from the Parylene membrane (red arrow) during preparation. The Parylene membrane is fabricated so that it contains thin areas (white bracket) and ultrathin areas (red bracket) where diffusion occurs more easily.

Ongoing preclinical studies from multiple groups, including ours, should provide data to support a future clinical trial using support scaffolds. Our group has focused on culturing RPE sheets on a scaffold of ultrathin Parylene membranes as mentioned above. Preclinical studies in the rat and minipig have been used to develop customized tools for implant delivery into the subretinal space. Also, the surgical technique has been adapted to an intraocular approach for humans, which consists of conventional pars plana vitrectomy with subretinal implantation of hESC-RPE through a retinotomy (Fig. 6).

Dr. Pete Coffey's group (University College London, London, UK) in collaboration with Pfizer (Tadworth, Surrey, UK) are working on transplanting hESC-RPE on a polyester scaffold. They are also studying iPSC-RPE on the same scaffold material. Drs. Kapil Bharti and Sheldon Miller at the NEI (NEI, NIH, Bethesda, MD) are focused on investigating polarized iPSC-RPE monolayers on biodegradable scaffolds (Bharti et al., 2014). Lastly, a group led by Dr. Massayo Takahashi (RIKEN, Wako-shi, Saitama, Japan) and Helios (Tokyo, Japan) has been investigating implantation of polarized human iPSC-RPE cells with no scaffold. A clinical trial has been initiated to test this approach in patients with advanced forms of NV-AMD. Although no clinical data have been published to date, Kamao et al. have recently published RIKEN's pre-clinical results in which human iPSC-RPE cells are cultured in sheets without any artificial scaffold. A type I collagen gel is used as a base on which the cells are seeded to form a human iPSC-RPE monolayer. After the cells reach confluence, collagenase is used to dissolve the collagen base. Results from both in vitro and *in vivo* tests indicate that human iPSC-RPE cell sheets show characteristics similar to those of native RPE (Kamao et al., 2014). P21 RCS rats received either 1 × 1 mm human iPSC-RPE cell sheet transplants or injections of human iPSC-RPE cells in suspension into the subretinal space. Eletroretinograms were performed 6 weeks later and a histological study 9 weeks later. In this study, no significant difference was reported between implantation of iPSC-RPE sheets and iPSC-RPE suspensions. Both groups showed improvement in ERG responses and a more preserved outer nuclear layer when compared with contralateral eyes. Additionally, the group prepared monkey iPSC-RPE cells from cynomolgus monkey (*Macaca fascicularis*) somatic cells and compared cell suspension injection to cell sheet transplantations in recipient monkeys. After 7 days, the injected cells were accumulated at the lower portion of the retinal detachment created for the subretinal injection, while transplanted cell sheets were at exactly the same place as delivered. Four monkeys that received cell sheets were observed for 1 year without immunosuppression. Monkey iPSC-RPE sheets were transplanted as allografts (with confirmed MHC mismatch) in three monkeys and as an autograft in one monkey. Monkeys that received allogeneic miPSC-RPE cell sheets showed typical signs of rejection, such as formation of fibrous tissues around the graft, leakage of fluorescein on angiography, and hyperreflective material under the retina or retinal edema around the graft on OCT. In contrast, the monkey with an autograft showed only slight hyperfluorescence around the graft, which was attributed to the artificial retinal detachment performed for the transplantation (Kamao et al., 2014).

8.3. Anatomical and functional outcomes of cell replacement therapies

8.3.1. Imaging studies

Advances in clinical imaging techniques, such as OCT, have enabled much better detection of early pathological changes in AMD. Also, this technological progress has allowed for the assessment of anatomical results in pre-clinical studies of cell replacement therapies. Although color fundus images are still important as a record of fundus examination at baseline and during follow-up, other imaging strategies are essential to evaluate clinical responses.

OCT is a very useful tool for the assessment of AMD, and consequently for the assessment of stem cell therapy of advanced forms of AMD. In the case of stem cell therapies utilizing cell suspensions, OCT images may show if a uniform layer of RPE is achieved. Follow-up OCT images confirm the final histological results after polarized hESC-RPE cells on Parylene scaffolds were implanted into the subretinal space (Hu et al., 2012).

In addition, confocal near-infrared fundus reflectance images, that are usually obtained at the same time of OCT scanning, have been shown to be efficient for detecting the presence of hESC-RPE cells *in vivo* following transplantation into the subretinal space

(Ribeiro et al., 2013). Fluorescein angiography (FA) can be used to assess retinal vasculature and early signs of immune response following stem cell transplantation.

8.3.2. Histological outcomes

In animal studies, examination of histology sections is crucial to establishing the location of transplanted cells and demonstrating proper subretinal placement of seeded membranes. The RPE cell monolayer should ideally remain intact after implantation, without retinal trauma or damage to the Bruch's membrane (Hu et al., 2012). Immunohistochemistry is used to evaluate survival of implanted cells in animal models, where different cellular markers are assessed to demonstrate the viability and functionality of the transplanted cells. Human cell marker (TRA-1-85), RPE-specific 65 kDa protein (RPE65), and epithelial cell markers (e.g. cytokeratin) are used as markers to identify human RPE cells in animal models. In addition, specific markers for rod photoreceptors (rhodopsin), rod bipolar cells (α-PKC), cone photoreceptors and bipolars (recoverin), ganglion and horizontal cells (RT97), and astrocyte/Muller cells (marker anti-GFAP) are used to assess the specific retinal cell types. Also, cell markers for lymphocytes (CD45), macrophages (anti-CD68), and other immune cells are used to search for the nature of immune reaction against the transplanted cells (Wang et al., 2008).

8.3.3. Functional assessment

As stem cell-based treatments for AMD pass preclinical safety studies and head towards clinical reality, measuring the preclinical efficacy of these treatments in animal models becomes increasingly important. Assessing visual function in animals requires objective measures to test visually-guided behaviors (Whiteley et al., 1996; da Cruz et al., 2007) as follows:

8.3.4. Electroretinography

Electroretinography (ERG) is used to determine the overall electrical response of the retina. A flash ERG response has been used to assess the visual function of animal models of retinal degeneration for years (Jiang and Hamasaki, 1994; Pinilla et al., 2005; Lund et al., 2006; Sauve et al., 2006; Krohne et al., 2012; Thompson et al., 2014). ERGs are recorded using contact electrodes placed on the surface of the animal's corneas during full-field light stimulation. Electrodes are also inserted into the forehead and tail as reference and ground electrodes, respectively. Scotopic ERGs are recorded under dark-adapted conditions and photopic recordings are performed after light-adaptation. The b-wave is normally accepted as a reliable measure of overall retinal function while the a-wave is generated more specifically by the photoreceptor cells. In RCS rats, the a-wave is commonly lost when they reach approximately P60, while the b-wave is usually severely decreased by P90 (Lund et al., 2006). The magnitude of response to an RPE implant in the rats can then be measured and compared to sham or untreated animals to evaluate the potential beneficial effects of the treatment for retinal degeneration. Lund et al.'s study revealed that hESC-RPE-injected RCS rats achieve significantly better a-wave, b-wave, and cone-mediated responses compared to untreated and sham-injected rats (Lund et al., 2006). A significant increase in rod and cone-driven ERG function is also observed with human umbilical cord cells transplanted in the subretinal space of RCS rats (Lund et al., 2007), mouse iPSC-photoreceptor precursor implantation (Tucker et al., 2011), and subretinal transplantation of rodent mesenchymal stem cells (Huang et al., 2013). Besides full field flash ERG, focal electroretinography (fERG) (Nistor et al., 2010) has also been performed to determine photoreceptor rescue in animal models. With fERG, it is possible to focus on a discrete region of the retina and determine if there is significantly more

electrical activity and photoreceptor rescue after iPSC-RPE injection in RCS rats (Krohne et al., 2012).

8.3.4.1. Optokinetic head-tracking (OHT) response. Spatial visual acuity can be assessed in animal models using a non-invasive behavioral testing, based on OHT response which was originally described by Cowey and Franzini (Cowey and Franzini, 1979; Lund et al., 2001a, 2001b; Coffey et al., 2002; Thaung et al., 2002). This is the most widely accepted method to test visual function (Seiler and Aramant, 2012). The OHT response is a compensatory eye movement that reduces movement of images across the retina (Harvey et al., 1997). Variations in OHT responses are affected by a number of factors including the population and distribution of surviving photoreceptors, the inner retina plasticity status, and the morphological status of higher visual areas of the brain such as the superior colliculus (SC). The OHT setup is comprised of four computer screens arranged in a square around the test animal with vertical black and white stripes of different widths displayed on them. This creates a virtual, three-dimensional (3D) space around the animal that rotates (i.e., the vertical stripes move) to stimulate head turning as the animal tracks the movement. Grating frequency (e.g. 0.04, 0.11, 0.22, 0.45, 0.89 cycle/degree) corresponds to acuity threshold. The images rotate slowly clockwise or counterclockwise while the unrestrained animal is placed in the center of the apparatus with a video camera placed over the platform for recording. Visual acuity is assessed by the highest frequency grating (the narrowest stripes) that the animal still tracks by turning its head (Lu et al., 2009; Coffey et al., 2002; Lund et al., 2001a, 2001b). Contrast sensitivity can also be determined by varying the contrast of the black and white stripes (Thomas et al., 2010). By varying the stimulus parameters, such as grating spatial frequency and rotational speed, it is possible to elucidate parameters that can evoke threshold OHT responses in animal models, and therefore to assess the outcome of stem-cell based therapeutic interventions. Previous research has found that subretinal injection of hESC-RPE (Lund et al., 2006), human umbilical cord cells (Lund et al., 2007), Human Central Nervous System stem cells (HuCNS-SC) (McGill et al., 2012), human bone marrow-derived mesenchymal stem cells (Wang et al., 2010), or somatic cells (Lu et al., 2010a, 2010b) (hBM-SCs) in RCS rats elicited higher levels of optokinetic response than in control (sham or untreated) groups.

8.3.4.2. Superior colliculus recording. Evaluation of the animal's ability to relay a light stimulus to the intermediate vision centers can also be conducted by invasive superior colliculus recording (Girman et al., 2003; Girman et al., 2005; Lund et al., 2006; Krohne et al., 2012; Thompson et al., 2014). The superior colliculus, located in the midbrain of mammals, receives direct retinal input that is topographically organized and corresponds to the areas of the retina that are stimulated by light (Siminoff et al., 1966) (Thomas et al., 2004). These visual signals from the retina can be recorded by observing the corresponding superior colliculus regions, and consequently a topographic map of receptive fields showing rescued or yet degenerated retinal areas can be generated. This activity in the superior colliculus can be easily monitored with the help of stereotactic coordinates after the superior colliculus is exposed after parietal craniotomy. Monitoring is performed using a microelectrode inserted into the superficial layers of the superior colliculus, and the luminance thresholds of visually-evoked responses triggered by a light flash projected onto the contralateral eye are recorded (Zhang et al., 2014). The test provides point-to-point estimates of retinal function, in a way similar to the visual perimetry test in humans. In RCS rats, the progression of retinal degeneration is reflected by a gradual decline of sensitivity in the superior colliculus from the temporal to the nasal visual field

(Sauve et al., 2002). Previous studies have demonstrated that transplantation with fetal retina sheets (Arai et al., 2004), hESC-RPE (Lund et al., 2006), human umbilical cord cells (Lund et al., 2007), bone marrow-derived mesenchymal cells (Otani et al., 2004; Lu et al., 2010a, 2010b), and HuCNS-SC (McGill et al., 2012) improve preservation of photoreceptor cells (i.e., visual rescue) and various parameters of SC response in rodent models of retinal degeneration.

8.3.4.3. Other tests. Other, non-invasive psychophysical tests, i.e. visually-guided behavior tests, utilized to assess the efficacy of retinal cell replacement therapies include water maze tasks, monitoring light–dark preferences, pupillary light reflex, and wheel running activities (McGill et al., 1988; Little et al., 1998; Kwan et al., 1999; Klassen et al., 2001; McGill et al., 2004). In the visual water maze test, the animals are trained to escape a pool of water by swimming to a platform hidden below the surface of the water. Depending on the ability of the animal to discern a stationary image (based on retinal input to the dorsal lateral geniculate nucleus, superior colliculus, and/or pretectum), the animal may or may not be able to see the platform when the actual experiment is conducted (Prusky et al., 2000; Thompson et al., 2014). A running wheel test assesses vision by the enhancement or suppression of wheel-running activity. It has been used to determine the lowest luminance that supports useful vision for the mobility of a mouse model (Thompson et al., 2008; Thompson et al., 2014). The pupil light reflex (PLR), or constriction of the pupil of the eye in response to light, involves the integrity of the reflex loop involving midbrain centers (Klassen and Lund, 1990; MacLaren et al., 2006). A locomotory light-avoidance assay and an open field test (Polosukhina et al., 2012; Tochitsky et al., 2014) have also been used to evaluate the changes in visual function in rodent models.

8.3.5. Safety studies and immune rejection

To ensure safe translation of hESC-RPE transplantation as a viable option for patients, a standardized strategy should be adopted to eliminate potential hazards associated with this type of cell treatment. Optimum safety and purity of the cells, and ability to act as true RPE cells, should be ensured before transplantation. In other words, stem cell-derived RPE cells should be free of undifferentiated cells and pathogens, and demonstrate the genetic and functional signature of RPE cells. This requires extensive testing for the absence of tumor formation and cell migration before attempting human implantation. Similarly, Good Manufacturing Practices (GMP) should be enforced and monitored during the clinical trials (Bharti et al., 2014).

Undifferentiated pluripotent stem cells, by definition, have the capacity to differentiate into all cell types of the three germ layers, which is why it is important to ensure that hESCs and iPSCs are completely differentiated before implantation, as undifferentiated cells may cause tumor formation (Fig. 7). Differentiation into non-desired cell types is also a potential threat to the success of stem cell-derived cell replacements. Lack of the ability to create a teratoma (a tumor composed of tissues of ectodermal, mesodermal, and endodermal origin) and other tumors is a major criterion for the safety of stem cell-derived cell therapies. This ability is assessed using tumorigenicity studies in animal models that investigate the formation of teratomas or other tumors following stem cell implantation. Because assessing tumorigenicity potential in immunocompetent animal models can be misleading since the absence of tumor formation might be related to the ability of the host to reject tumorigenic cells before tumors form, various immunodeficient models are used instead such as the athymic nude rat, CD-1 Nude Mouse, NU/NU Mouse, and BALB/c Nude Mouse when studying the tumor formation potential of hESC-RPE and iPSC-RPE cells. The athymic nude rat, characterized by a lack of normal thymus (and, thus, a lack of functionally mature T cells), is frequently used in the study of tumor growth or graft rejection (Diniz et al., 2013). However, other functional cells of the immune system such as macrophages, natural killer cells, and B lymphocytes may contribute to the innate immune response to non-self cells and, consequently, to the killing of tumor cells (Preynat-Seauve et al., 2009).

Given that the risk of tumor formation by contaminating undifferentiated cells is still being investigated, confirming the purity of hESC-RPE and iPSC-RPE cell populations for transplants is mandatory. One study has shown that at least 0.6% of mouse iPSC cells remained undifferentiated up to 15 days after iPSC cells were transferred to a serum-free, feeder-free suspension culture condition designed to differentiate to RPE cells (Hirami et al., 2009). However, Kuroda and co-workers have reported that "human iPS cell-derived RPE cells after 4 passages showed no detectable contamination of immature cells (Kuroda et al., 2012). Some methods have been suggested to remove the contaminating undifferentiated cells from stem cell-derived grafts before transplantation. Human serum normally contains antibody against α-gal (galactose-alpha-1,3-galactose) epitopes expressed in all non-primate mammalian cells that renders us immunoreactive to dietary and gut flora sources of α-gal. This antibody is responsible for immediate rejection of organs from non-primate mammals transplanted into the human body. To remove the undifferentiated pluripotent stem cell population from the differentiated cell pool, Hewitt et al. engineered hESC to express α-gal and showed that upon differentiation, these cells lose their α-gal epitopes and upon
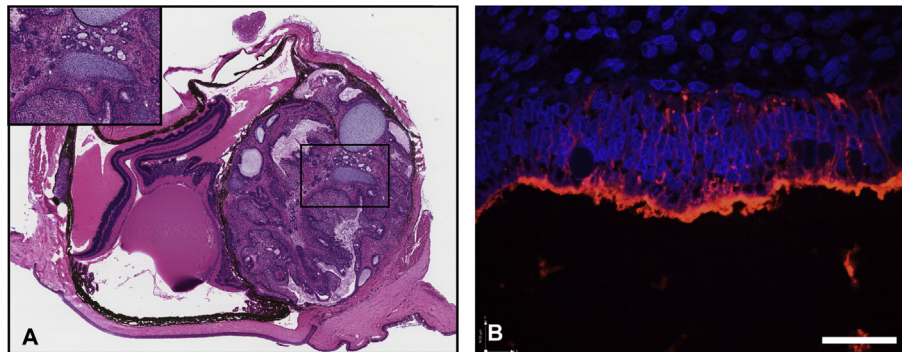
Fig. 7. Teratoma formation due to transplantation of *undifferentiated* human embryonic stem cells (hESCs). (A) Teratoma formation after subretinal injection of undifferentiated H9 hESCs into the subretinal area of a nude SCID rat. Area marked in A is magnified in inset to show cells with epithelial, mesenchymal, and endothelial origin within the teratoma. (B) Cells are stained for human cell marker TRA-1-60 (red), showing that the teratoma cells are from human origin (scale bar: 20 μm).

exposure of hESC-derived cells to active human serum, the undifferentiated fraction of the cells die because of antibody dependent cell cytotoxicity. The authors suggested that not only can this method remove undifferentiated cells before transplantation, it could also provide ongoing immune surveillance after engraftment against undifferentiated or dedifferentiation stem cells (Hewitt et al., 2007).

Animal toxicology, safety, and efficacy studies provide information necessary for proceeding to clinical trials. These preclinical animal studies are specifically needed to determine the potential expression of known proto-oncogenes or tumorgenesis risk. Pathological analyses of these studies should be applied to retina tissue as well as whole animal body organs in case cells migrate from the retina. Also, the surgery procedure should be carefully assessed to mimic the procedure to be used in humans and minimize possible surgical complications.

Finally, there is growing concern about potential epigenetic remnants in both differentiated hESCs and iPSCs including cancerlike hypermethylation of promoter DNA, pluripotency-associated histone markers, and aberrant regulation of X-chromosomes (Huo et al., 2014). These genetic aberrations may increase the risk of dedifferentiation drift of the final therapeutic cell lineage (i.e., tumor formation). Use of efficient, high-fidelity reprogramming derivation methods has been suggested to minimize cancerassociated epigenetic aberrations in iPSCs (Huo et al., 2014). Modifications of differentiation protocols (Tomoda et al., 2012) and in vitro detection of undifferentiated cells by flow cytometry assays using an anti-TRA-1-60 antibody and/or qRT-PCR to detect trace amounts of Lin28 mRNA have also been suggested to ensure detection of potential tumor-producing cells during the production of hESC-RPE and iPSC-RPE cells (Kuroda et al., 2012).

9. Ongoing research and clinical trials

Stem cell based-treatment for retinal degeneration disease is currently transitioning from over a decade of basic research to preclinical and clinical trials (Bharti et al., 2014). Several studies have reported that subretinal transplantation of hESC-RPE results in improvement of vision, and significant photoreceptor rescue, without deleterious side effects (Lu et al., 2009; Lund et al., 2006; Schwartz et al., 2012, 2015). To date, there are at least 14 ongoing clinical trials testing stem cell-based replacement therapies for the treatment of macular degeneration registered in the International Clinical Trials Registry Platform (ICTRP) of the World Health Organization as of March 1, 2015 (Table 4). Also, in July 2013, Japan's Ministry of Health approved the first clinical study using RPE derived from iPSCs for the treatment of AMD. All of these ongoing trials and clinical studies are based on either autologous or allogeneic transplantation of several kinds of stem cells, including hESC-RPE, iPSC-RPE, hUTSC, BMHSC, HuCNS-SC, and Adipose-Derived Stromal Cells. Of these, hESC-RPE and iPSC-RPE replacement therapy trials seem to be the more promising based on the available publications and reports. In addition to AMD, target diseases for such cell replacement therapies encompass retinitis pigmentosa, hereditary retinal dystrophies such as Stargardt's disease, optic nerve disease, diabetic retinopathy and retinal vein occlusion. A variety of cell delivery methods, including subretinal transplantation with cell suspensions or cell sheets, as well as intravitreal injections, are currently being used. The majority of these trials are phase I or phase I/II trials in their early stages of enrollment. The projected number of participants in most of the current clinical trials is less than 100, with the maximum number being up to 300 in a multiple cooperative study sponsored by Retina Associates of South Florida and MD Stem Cells. Since the focus of this review is on human embryonic and induced pluripotential stem cell derived RPE, below, we summarize the current 4 major hESC-RPE and iPSC-RPE trials. We have also included a trial testing human central nervous system stem cells in this list.

9.1. Ocata Therapeutics trial using subretinal hESC-RPE suspensions

Ocata Therapeutics, Inc., formerly named Advanced Cell Technology, has sponsored the first phase I/II, open-label, multi-center, prospective study to determine the safety and tolerability of subretinal transplantation of hESC-RPE (specifically line MA09-hRPE) cell suspensions in patients with advanced dry AMD and Stargardt's disease. Following the first trial of subretinal delivery of 50,000 hESC-RPE MA09 cells in two patients with AMD and Stargardt's disease (Klimanskaya et al., 2006), the trial to enroll patients with advanced dry AMD (NCT01344993) is being conducted at four centers in the U.S.: University of California, Los Angeles Stein Eye Institute (Los Angeles, CA); Wills Eye Hospital (Philadelphia, PA); Bascom Palmer Eye Institute (Miami, FL); and Massachusetts Eye and Ear Infirmary (Boston, MA). The treatment procedure and outcomes were published for 18 patients, including 9 patients with advanced dry AMD and 9 patients with advanced Stargardt's disease (Schwartz et al., 2015). The worse eye of each patient (with a BCVA of equal or less than 20/400 or 20/400–20/100) was used for the trial. Three dose cohorts (50,000, 100,000, and 150,000 hESC-RPE cells), with 3 patients in each cohort, were treated with subretinal injection of the cell suspension following vitrectomy and induction of posterior vitreous detachment. Specifically, 150 μL of an hESC-RPE cell suspension was injected through a MedOne PolyTip Cannula (23G/38G or 25G/38G), delivering the intended dose of hESC-RPE cells into the transitional zone of the retina, at the area between atrophic retina/RPE/choriocapillaris and relatively healthy post-equatorial retina. OCT is used to confirm subretinal injection of hESC-RPE suspension in the reconstruction of the RPE layer (Schwartz et al., 2012, 2015). The immunosuppression regimen included tacrolimus and mycophenolate mofetil starting 1 week before the surgery and continuing for approximately 12 weeks. As the main endpoint, the trial cohort was assessed for the safety and tolerability of subretinal hESC-RPE suspension transplantation. The secondary endpoints were the efficacy of hESC-RPE in preserving or improving vision as assessed in serial ophthalmic examinations. The patients underwent follow-up examinations for a median of 22 months (Schwartz et al., 2015).

The authors reported no evidence of adverse proliferation including macular pucker, tumor formation, rejection, or other serious ocular or systemic safety issues related to the transplanted RPE tissue itself. However, adverse events possibly associated with immunosuppression were seen in almost all patients. Patches of increasing subretinal pigmentation consistent with transplanted RPE were documented in 13 out of the 18 patients (72%). Best-corrected visual acuity improved in 10 eyes (56%), improved or remained the same in 7 eyes (39%), and decreased by more than ten letters in 1 eye (5%). Untreated fellow eyes did not show any visual improvement. Vision-related, quality-of-life measures increased for general and peripheral vision, and near and distance activities. Despite the small size of the patient population in the study, the results have provided strong evidence of the medium-to long-term safety, graft survival, and possible biological activity of injection of hESC-RPE cell suspensions in individuals with AMD. This suggests that hESC-RPE cells could provide a potentially safe new cell source for cell replacement therapies (Schwartz et al., 2015). Given the drawbacks of subretinal hESC-RPE cell suspension transplantation compared to transplantation of a polarized hESC-RPE sheet (sections 8.2.1 and 8.2.2), long term follow up reports are needed to support the potential for such transplants in restoring degenerated RPE layer and promoting PR survival.

9.2. Regenerative Patch Technologies (RPT)-subretinal hESC-RPE sheet

A clinical trial is being planned by Regenerative Patch Technologies (RPT) to explore the safety and efficacy of subretinal implantation of hESC-RPE monolayers seeded on an ultrathin Parylene membrane at the University of Southern California Eye Institute. This trial, supported by the California Institute for Regenerative Medicine (CIRM), is the result of a collaboration between investigators at several institutions, including the University of Southern California, City of Hope, Caltech, and the University of California, Santa Barbara, to derive and characterize monolayers of RPE cells derived from H9 hESC. The ultrathin Parylene scaffold used in this study supports hESC-RPE cells as an artificial Bruch's Membrane that allows free transport of water and small molecules through the artificial membrane and provides a platform for hESC-RPE cells to attain a uniform, differentiated monolayer (Lu et al., 2012). The preclinical safety and efficacy of hESC-RPE implants have been demonstrated by implantation studies in RCS rats and the implantation surgical techniques refined in Yutacan pigs as outlined above. A human phase I/IIa clinical trial will explore the safety and tolerability of subretinal hESC-RPE implants in patients with GA involving the central fovea. Two cohorts of patients with GA and BCVA of ≤20/400 and ≤ 20/100->20/400 are planned to be recruited. The patients will be immunosuppressed for a brief period (~2 months) and gradually tapered off immunosuppression in the absence of any signs of immune response. In addition to fundus examination, subretinal placement of the implant and retinal layer integrity and thickness will be confirmed using OCT immediately after the implantation and during the follow-up period.

9.3. London project to Cure Blindness-subretinal hESC-RPE sheet implantation

University College London is approved to conduct the first trial to recreate the natural anatomy of the RPE in patients with acute vision loss because of NV-AMD. The London Project to Cure Blindness (LPCB), sponsored by Pfizer, has developed an hESC-RPE cell line that can be cultured as a monolayer on a thin sheet of polyester membrane for this trial. A phase 1 study will start in 2015 to assess the safety of subretinal transplant of the hESC-RPE monolayer immobilized on the membrane (Carr et al., 2013) in 10 patients with wet AMD and rapid recent vision decline (NCT01691261). The polyester membrane will be approximately 6 mm × 3 mm. Intraocular corticosteroids will be used for immunosuppression. LPCB is planning another study to treat patients with AMD, specifically ones with RPE tear, using iPSC-RPE monolayers on a polyester scaffold. Although the polyester film has been tested as a substrate for the culture of RPE cells for cell replacement therapies (Stanzel et al., 2012; Stanzel et al., 2014), the long-term survival and functionality of iPSC-RPE and hESC-RPE on polyester membranes and long-term transport of water, oxygen, and nutritional and waste molecules through such membranes are not fully clear (Stanzel et al., 2014).

9.4. RIKEN-subretinal iPSC-RPE sheet implantation

In July 2013, Japan's health minister approved the first clinical study using cells derived from iPSCs. A preclinical study showed the phagocytotic ability and gene-expression patterns of the iPSC-RPEs to be similar to those of native RPE (Kamao et al., 2014). Six patients with subfoveal CNV, fibrotic scar, or RPE tear (related to exudative AMD) with a BCVA of less than 0.3, but better than hand motion, will be enrolled and followed for up to 4 years. For this study, iPSCs are generated from a patient's skin, differentiated to iPSC-RPE and then cultured as an iPSC-RPE monolayer on a collagen gel initially used as a temporary scaffold. The size of the RPE sheet used in the study will be at least 1.3 mm × 3 mm. The first iPSC-RPE cell sheet was transplanted to a 70-year-old female with AMD in September 2014; no further details about visual acuity have been reported yet (http://www.riken-ibri.jp/AMD/).

9.5. StemCells, Inc.-subretinal HuCNS-SC suspension implantation

A phase I/II study (NCT01632527) of the safety and preliminary efficacy of subretinal injection of HuCNS-SC in AMD with GA is ongoing by StemCells, Inc. The trial is based on a pre-clinical study showing that human neural stem cells derived from the fetal brain and grown as neurospheres (HuCNS-SC) can protect host photoreceptors and preserve visual function after transplantation into the subretinal space of P21 RCS rats (McGill et al., 2012). StemCells, Inc. sponsored the study to determine the long-term safety and possible benefits of subretinal injection of HuCNS-SC for patients with GA/AMD. Patients with BCVA of 20/400 were enrolled in Cohort I. 2×10^5 cells were injected in each of 4 patients, and 1×10^6 cells injected in each of 4 additional patients. Cohort II enrolled patients with BCVA of 20/320–20/100 in 7 patients which were each injected with 1×10^6 cells. Patients were given oral immunosuppressive agents for 3 months after surgery and followed up for 1 year. A long-term follow-up of 8 patients of the same study is currently ongoing for an additional 4 years (NCT02137915). The interim data based on a minimum of 6 months follow-up (http://www.stemcellsinc.com/) have shown maintenance or improvement in BCVA and contrast sensitivity with no safety concern about HuCNS-SC.

10. Major challenges and concerns

Major hurdles in bringing stem cell-based therapies to the bedside are the cost, time, and labor-intensive nature of the process (Bharti et al., 2014). While cost is an even greater issue for autologous transplantation (iPSC-RPE), the allogeneic transplantation cost needs to be decreased as well. The process, from initially harvesting somatic cells to preparing a ready-to-use iPSC-RPE implant, takes 9–12 months (Jin et al., 2009; Kuroda et al., 2012; Kamao et al., 2014). It is possible that creating a bank of cell lines of iPSC-RPE, with known surface antigen markers, would eventually decrease the cost of production and offer an off-the-shelf treatment that can be used once a patient is designated as a candidate for such therapies.

10.1. Long-term safety

Although methods used to differentiate RPE from stem cells do not support survival of stem cells, a major concern with stem cell-derived RPE and RPC replacement therapies as pointed out above is whether a small number of stem cells may remain in the transplanted cells and cause tumors. While preclinical safety data, including toxicology and tumorigenicity analysis, can theoretically isolate undifferentiated cell populations and rule out the risk of tumor formation after transplantation of differentiated cells such as hESC-RPE and iPSC-RPE (Kuroda et al., 2012; Kanemura et al., 2014), one cannot confirm how these cells will actually behave when implanted into the eye. In this regard, key limitations in preclinical study publications should be considered when discussing the tumorigenicity issue with patients. In addition, most animal studies are relatively short-term (i.e. 6–9 month) and it is not clear what the tumorigenicity potential in long-term would be. The first 3–5 years of human trials with stem cell-derived RPE and RPC replacement will be especially important regarding this concern. It

should be remembered that cell replacement therapy for AMD has the advantage that it is easy to detect any abnormal growth in the eyes and, because of the encapsulated nature of the eye structure, less likely that cells can escape the eye and form tumors elsewhere in the body. Another major safety challenge to be addressed is the immune reaction to autologous and allogeneic stem cell-derived RPEs (section 7).

10.2. Long-term function/survival of the implanted cells

One of the first determinants of whether stem cell-derived RPE survives and functions properly in the subretinal space is whether the cells injected into the subretinal space in suspension adhere to the underlying Bruch's membrane and integrate into the overlying photoreceptors outer segments. Given the extent of abnormality in aged Bruch's membrane in a patient with advanced AMD (Sugino et al., 2014), reliably spreading hESC-RPE cells (delivered in suspension) across the Bruch's membrane to create an adherent monolayer is a challenge. Ultrathin Parylene membranes (Hu et al., 2012; Diniz et al., 2013), plasma polymers (Kearns et al., 2012), biodegradable polyester membranes (Stanzel et al., 2012), polyamide nanofibers (Li and Tang, 2012), biopolymers coated with polyimide (Subrizi et al., 2012), polytetrafluoroethylene (Kearns et al., 2012), and polyester matrix (Blenkinsop et al., 2013) are among the materials suggested to be used as platforms on which to seed RPE monolayers and act as a suitable artificial Bruch's Membrane. As stated before, two clinical trials with stem cell-derived RPE cells cultured on Parylene and non-biodegradable polyester membranes are planned to be initiated in late 2015.

10.3. Functional consequences of stem cell-derived RPE implantation

Preclinical data obtained from the functional effects of cell replacement therapies support the role of this treatment in both stopping the progression of retinal degeneration and regenerating the retinal elements. Multiple reports show that deterioration of cone and rod photoreceptors is halted after transplantation of various RPE cell lines, including iPSC-RPE and hESC-RPE into the subretinal space (Li and Turner, 1991; Little et al., 1996; Girman et al., 2005; Pinilla et al., 2007; Wang et al., 2008; Park et al., 2011). RPCs are also shown to integrate into the retina of animal models. Hambright et al. injected hESC-RPC into the vitreous cavity and into the subretinal space and showed that, while limited synaptogenic activity was observed, hESC-RPCs integrated into the mouse photoreceptor layer when injected into the subretinal space and these cells integrate into the mouse ganglion cell layer and inner nuclear layer when injected into the vitreous cavity (Hambright et al., 2012). Not only the physical proximity, but also intrinsic signals from the local extracellular matrix and neighboring cells, are proposed to determine how RPCs integrate and regenerate the host retina (Qiu et al., 2005; Gong et al., 2008). Although the degenerative process may continue to affect remaining RPE and retinal cells, growth factors secreted from newly implanted RPE or RPC cells may help to slow down this degenerative process. Long term reports from clinical trials may prove whether such implantations actually slowed the progression of degeneration in close-by, non-implanted areas of the retina.

10.4. Optimal timing of stem cell-derived RPE implants

A surprisingly good functional outcome can be achieved after autologous transplantation of RPE-choroid sheets in patients with advanced AMD in whom RPE and retina were severely damaged (Binder et al., 2004; Treumer et al., 2007; Heussen et al., 2008; van Zeeburg et al., 2012; Han et al., 2013). Currently, stem cell-derived

RPE transplantation trials are performed at late stages of retinal degeneration because of the nature of the initial phase I trials which are designed for the assessment of the safety of such treatments rather than their efficacy. The first patient with AMD who received an hESC-RPE injection into the subretinal space had a BCVA of 21 Early Treatment Diabetic Retinopathy Study (ETDRS) letters (equal to 20/500) (Schwartz et al., 2012). The iPSC-RPE trial in Japan performed on a 70-year-old female with AMD has not released details about visual acuity (http://www.riken.jp/en/pr/topics/2014/20140912_1/), ideally, such implantations should be performed in patients with higher potential for visual rehabilitation. So far, the optimal timing for initiation of stem cell-based treatment of AMD in order to achieve the best outcome while balancing the risk of surgery and perioperative care is not clear at all. With an early transplantation, a higher number of photoreceptors may be available for rescue and thus a better outcome for the patients. On the other hand, an initial, possible dramatic drop in vision can be expected with early treatments where the starting vision is not as low. To define a threshold for hESC-RPE or iPSC-RPE transplantation, the following considerations should be stressed. First, because advanced forms of AMD occur predominantly in ages 80 and older, transplant-related complications could be relatively high in that age group. Second, AMD may progress slowly over many years, and a surgical transplant procedure at an early stage may be controversial, considering that it subjects the patient to significant morbidity for a longer time. Third, although some animal studies support the regeneration of vision even with late treatment (Wang et al., 2008), it is still unknown whether vision can be restored in a severely degenerated retina with long-term photoreceptor loss. In addition, once photoreceptors are lost in advanced stages of degeneration, cell replacement treatment would have to involve combined transplantation of photoreceptor and RPE cells, which has not yet been achieved to a level sufficient to consider clinical trial approval. Fourth, emerging improvements in cell-based therapies, as well as in new non-transplant or pharmacological strategies, for early AMD may affect the timing considered optimal for the cell-based therapies. Fifth, the disease does not progress with the same speed in all patients, so treating a patient with rapid progression of the GA area with a certain level of VA may have to be quite different for a patient with the same VA but with very slow progression of the disease (Klein et al., 1997). A recent study demonstrated that development of foveal scars, pigmentary abnormalities, or GA contributed to most of the sustained visual acuity loss and had poor final visual acuity outcomes (Ying et al., 2014). Patients with such features have a higher chance of losing vision because of AMD and may have a lower threshold for consideration of hESC-RPE/iPSC-RPE transplantation.

11. Future trends in stem cell-based treatment for macular degeneration

As suggested in an NEI workshop, a collaboration on various aspects of translating stem cell science to clinical use, including clinical grade cell lines, reporter iPSC lines, SC to SC-RPE differentiation protocols (research grade or clinical grade), transplantation tools, and drug master files (DMF), is needed to expand stem cell science and its translation into the clinic (Bharti et al., 2011). In addition, it seems that the involved companies and research groups are interested in advancing the following approaches in stem cell-based cell therapies in the eye as outlined below:

11.1. Combined RPE-Retinal progenitor cell replacement therapy

As stated before, the stem cell-based cellular therapeutics for AMD are mainly focused on (1) replacing RPE to maintain normal

function such as phagocytosis or (2) RPC replacement to regenerate lost retinal elements, including photoreceptors. However, for the patients with extensive photoreceptor cell death, focus will need to on replacing the photoreceptor cells in conjunction with the RPE graft. Klassen et al. demonstrated that RPCs grafted into the degenerating retina develop into mature neurons, including presumptive photoreceptors expressing recoverin, rhodopsin, or cone opsin, along with widespread integration of donor cells into the inner retina (Klassen et al., 2004). Nistor et al. first developed a 3D construct of RPE and RPCs from hESCs (Nistor et al., 2010). The 3D retina-RPE tissue was constructed by culturing hESC-derived neural retinal progenitors in a matrix on top of hESC-RPE cells in a cell culture insert fed by an osmolarity gradient (Nistor et al., 2010). The product of the hESC-derived tissue constructs expressed markers characteristic of retinal ganglion cells, photoreceptor development, and other neuronal cells (Nistor et al., 2010).

A major challenge in developing retinal tissue (with or without RPE combination) is re-establishing afferent nerve connections to the proximal retinal elements. The most rudimentary types of such connections are short, single synaptic connections to the remaining inner retinal circuitry. A more advanced signal processing would necessitate the establishment of more complex connections with neural circuitry of the host, including but not limited to bipolar, amacrine, and Muller cells (MacLaren et al., 2006).

11.2. Combining stem cell and gene therapy

Gene therapy is another treatment modality in which stem cells will likely play a significant role in the near future. Stem cell implantation, combined with gene therapy, has been performed in the treatment of nonocular genetic disorders for years (Rideout et al., 2002; Hanna et al., 2007; Sergijenko et al., 2013). hESCs can be genetically manipulated to introduce a therapeutic gene, either active or awaiting later activation once the modified hESC has differentiated into the desired cell type. iPSCs made from a patient's own cells may replace a faulty disease gene itself with a normal, healthy copy. The repaired stem cells could then be directed to form the tissue type needed, delivered into the eye, and used to reconstitute the diseased tissue (Simara et al., 2013). Restoration of normal gene function in iPSC cultures could be attainable using viral vectors that are currently being employed in human gene therapy trials, provided that the target gene has been appropriately packaged and expressed. The self-renewal ability of stem cells also suggests that there is a reduced, or absent, need to provide repeated administrations of gene therapy and better maintenance of therapeutic effects.

In a recent report, homologous recombination to correct the genetic defect in iPSCs derived from a patient with retinal gyrate atrophy allowed for expansion of iPSC with a corrected gene in which transplantation of iPSC-derived cells did not increase genomic instability (Howden et al., 2011). Another study of MSC transplantation with erythropoietin gene modification in a rat retinal degeneration model demonstrated an elevated EPO concentration in vitreous and retina (Guan et al., 2013), suggesting the therapeutic prospects of combined stem cell and gene therapy in AMD. In addition, genetically-modified human neural progenitor cells can be used as a proof of concept for the feasibility of combined stem cell and gene therapy for retinal diseases. In this regard, a lentiviral construct was used to generate human neural progenitor cells (hNPC) secreting glial cell line-derived neurotrophic factor (GDNF) that, in turn, was shown to augment retinal function and cone-specific electrical activity in the retina of RCS rats at P100 (Gamm et al., 2007).

This evidence shows that stem cells hold great potential for successful use in gene therapies in the clinic (Lipinski et al., 2013).

However, AMD is associated with multiple genetic risk factors and, thus, genetic manipulation of iPSC-RPE might not be a practical approach for such a disease.

11.3. Tissue engineering

As described above, implantation of cells cultured on thin polymer films could provide a means of transplanting an organized sheet of RPE cells with distinct apical/basal characteristics for the restoration of normal RPE function *in vivo*. Evidence has shown that using a polymer scaffold could offer better manipulation of the extracellular environment and afford the most appropriate degree of cell attachment, differentiation, and maintenance of apposite function (Tomita et al., 2005; Treharne et al., 2011; Kador and Goldberg, 2012; Kundu et al., 2014). Current ongoing preclinical trials are applying various types of scaffolds for monolayer stem cells transplantation. As mentioned above, our research has demonstrated successful delivery of an hESC-RPE monolayer into the rat's subretinal space on an ultrathin Parylene scaffold (Hu et al., 2012; Diniz et al., 2013), leading to initiation of a clinical trial.

Scaffolds can be either biodegradable or nonbiodegradable. Biodegradable poly (D,L-lactic-co-glycolic acid) (PLGA) and poly (L-lactic acid) (PLLA) are the most commonly studied materials in this area (Hadlock et al., 1999; Lu et al., 2001a, 2001b; Lu et al., 2001; Klassen, 2006; Thomson et al., 2010). These materials have the advantage of maintaining a differentiated cell phenotype, feasible for controlling RPE cell morphology and function. It has been demonstrated that thin biodegradable PLGA films can provide proper substrates for human stem cell-derived RPEs and photoreceptor cells (Nadri et al., 2013a, 2013b). The degradation rate of PLLA and PLGA can be manipulated by changing the molecular weight and the ratio of lactic to glycolic units; this can help to design the most appropriate timescale for degradation. Other polymers and preparation techniques have also been investigated for suitable biocompatible and mechanical properties (Table 5). Many factors, such as surface chemistry and surface topology, could affect the practicability of different materials for cell attachment and survival. Specific surface modifications aid in the survival of transplanted cells by mimicking their natural environment and these modifications have been studied in stem cell transplantations (Table 6). In addition, surgical techniques and new instrumentation for the subretinal implantation of biomaterial carriers of stem cells or RPE are also being actively explored (Stanzel et al., 2012)

Tissue engineering thus shows immense promise and potential in the development of treatments for AMD by employing scaffold-based delivery systems of RPE and RPC into the subretinal space. However, a major challenge remaining is the ever-present potential for increased macrophage activity in response to the presence of artificial material. Activated macrophages could increase the likelihood of rejection of co-grafted foreign cells (Klassen, 2006). More effort still needs to focus on scaffold thickness, surface topography, mechanical properties, and degradation characteristics, and to find the most efficient way to sustain viability and functionality of stem cell-based AMD treatment.

11.4. Mobilizing tissue-specific stem cells

It has been shown that a small population of cells in the human retina demonstrates stem cell-like characteristics of proliferation, self-renewal, and multipotentiality (Coles et al., 2004). Van der Kooy's group first dissected and cultured different subregions of human eyes (ranging from postnatal to old-age) and showed that stem cells could be derived from the pars plicata and pars plana regions at a frequency of 0.2%. To test the functional potential of

Table 5
Substrates for hESC-RPE, iPSC-RPE, and RPC implants.

Substrate material	Biodegradability	Potential advantage	Selected reference
Parylene	Non-biodegradable	Good mechanical strength, biostability, chemical inertness, supports RPE growth and polarization. Submicrone ultrathin areas are permeable to water, nutrients, and RPE waste products.	Lu et al., 2012
poly (glycerol sebacate) (PGS) scaffold	Non-biodegradable	Support RPC survival and express mature retinal proteins in a host retina	Wang et al., 2003, Redenti et al., 2009
poly (e-aprolactone) (PCL) nanowire scaffold	Non-biodegradable	Support RPC survival and express mature retinal proteins in a host retina	Redenti et al., 2008
Ultra-thin poly (methyl methacrylate) (PMMA) scaffold	Non-biodegradable	Contain pores that help to retain adherent RPCs to a considerably greater extent than un-machined versions during the transplantation process, and these can serve as a biocompatible substrate for cell delivery *in vivo*	Tao et al., 2007
Cross-linked gelatin scaffold	Non-biodegradable	Can be used as cell sheet carriers, with effective delivery of RPE sheets to the subretinal space	Rose et al., 2014
Hyaluronic acid (HA)-based hydrogels	Non-biodegradable	Degradable material for retinal progenitor cell (RPC) transplant	Liu et al., 2013
poly (D,L-lactic-co-glycolic acid) (PLGA)	Biodegradable	Degradable substances that may allow better transport of water and molecules across the RPE layer. Proposed as a better means for delivering RPCs to the subretinal space	Hadlock et al., 1999 Lu et al., 2001a, 2001b Lavik et al., 2005
poly (L-lactic acid) (PLLA)	Biodegradable	Degradable substances that may allow better transport of water and molecules across the RPE layer. Proposed as a better means for delivering RPCs to the subretinal space	Hadlock et al., 1999 Lu et al., 2001a, 2001b Lavik et al., 2005

Table 6
Specific surface modifications to enhance hESC-RPE, iPSC-RPE, and RPC transplantation results.

Surface Modification during the microfabrication process	Potential advantage	Selected reference
Ultrathin mesh like parylene membrane	Provides stablity to the membrane while keeping ultrathin areas for enough permeability to water and nutrients	Lu et al., 2012
oxygen plasma of the scaffold surface	To modify scaffold surfaces to generate a more hydrophilic surface	Tezcaner et al., 2003
Pre-activated, MMP2-loaded PLGA polymers	Can stimulate removal of the inhibitory ECM barrier and enhance stem cell integration and retinal repopulation	Tucker et al., 2010
Micropatterned surfaces (created using a microcontact printing technique)	Can also provide control of RPE cell morphology	Lu et al., 2001a, 2001b
PLGA scaffolds with fiber topography	Enhanced subretinal biocompatibility	Liu et al., 2014
Small intestinal submucosa PLGA hybrid films	Higher cell survival rate and much stronger phenotype expression compared to pure PLGA films	Lee et al., 2014

such stem cells, dissociated retinal spheres supposedly containing retinal stem cells were transplanted into NOD/SCID mice where they were able to survive, migrate, integrate, and differentiate into different retinal layers, including photoreceptors (Coles et al., 2004). In addition, Temple's group showed that a subpopulation of adult human RPE cells can be isolated and demonstrate self-renewal properties, lose RPE markers, and redifferentiate into stable, cobblestone, pigmented cells in a monolayer with other RPE characteristics. This study established RPE stem cells (RPESCs) as a human CNS-derived stem cell type that can be isolated and used for cell replacement therapy, disease modeling, and developmental biology studies (Salero et al., 2012; Blenkinsop et al., 2013). Whether regenerative mechanisms similar to those seen in fish and frogs that can repair the damaged retina are present in human retina and can be mobilized to repair the damaged retina is yet to be determined. In general then, there is "intrinsic regenerative potential of the retina using endogenous retinal cells" as Jeon and Oh point out in a recent publication. RPE cells, ciliary pigment epithelial cells as well as Muller glial cells can be used for this purpose (Jeon and Oh, 2015). Zhao and collaborators have demonstrated that Muller glial elements can be used for retinal repair and regeneration, being able to be reprogrammed into a retinal neuronal fate (e.g., photoreceptor cells) (Zhao et al, 2014). Suga and coworkers though have reported that the proliferation potential of Muller glial cells can differ substantially between different mouse strains and have shown that different sets of genes are upregulated in the retinal after damage (Suga et al., 2014).

12. Conclusion

Age-related macular degeneration and other macular degenerative diseases, such as Stargardt's Disease, are collectively the leading causes of retinal blindness with currently limited available therapeutic options. As RPE cell defects are implicated in the pathogenesis of many of these diseases, reestablishment of a healthy RPE layer through implantation of stem cell-derived RPE cells has created hope for treating blindness in millions of patients. Animal studies have been promising by showing the functionality of subretinally-implanted hESC-RPE cells and reasonable safety of this approach. Recent publications on human subject trials have reported good safety outcomes and provided grounds to support further tests of non-polarized and polarized hESC-RPE transplantation. The use of iPSC-RPE is also under trial to test safety and efficacy. Retinal progenitor cells and neural stem cells have also shown photoreceptor rescue upon intravitreal injection in animal models of macular degeneration. For all these stem cell-based treatments, safety, long-term functionality, cost, and technical complexity of the procedure, and optimum treatment timing are among unanswered challenges. The next few years will be exciting because of increased funding opportunities from institutions such as the National Eye Institute and the continued support of the California Institute for Regenerative Medicine and New York Stem Cell Foundation as well as the emergence of data from multiple stem cell-based retinal clinical trials, each of these having different approaches. However, a high level of commitment for collaboration and sharing of results is

needed to ensure that the huge investment in the field yields tangible results. As pointed out by the NEI, technical and logistical road blocks should be identified and potential solutions for clinical application, production, and regulation of stem cell-based therapies for retinal degenerative diseases should be found through collaboration of academic labs and private companies interested in this field (Bharti et al., 2011).

Acknowledgments

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