

Springer title: **Retinal Pigment Epithelium in Health and Disease**

Chapter title: **RPE and Stem Cell Therapy**

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ABSTRACT

Retinal pigment epithelium (RPE) is vitally important for supporting photoreceptor functions and welfare. RPE degeneration has a major role in pathogenesis of many retinal diseases including wet and dry forms of age-related macular degeneration (AMD). The loss of RPE cell functions leads to the degradation of photoreceptors and as a consequence to either partial or total loss of vision. Today, only cell replacement can restore RPE functionality, setting high demands for development of cell transplantation therapy. Transplantation of RPE cells derived from fetal, cadaveric, and stem cell sources have been extensively studied in different animal models and also in recent clinical trials. Based on current knowledge, human pluripotent stem cells (hPSC), including human embryonic stem cells (hESC) and human induced pluripotent stem cells (hiPSC), provide an exhaustless source of cells for cell-based therapies. Since 2004, numerous research groups have reported successful differentiation of functional RPE cells from hPSCs (hPSC-RPE) using a wide variety of methods. Two RPE cell transplantation strategies—each with their own pros and cons—are currently under development: injection of a single-cell suspension, and transplantation of intact RPE sheet with or without a biomaterial-based scaffold. So far, the ongoing clinical trials with hPSC-RPE have not raised any safety concerns, but additional proof of efficacy needs further trials. In light of the recent advances and the exponential activity in the field, this should be possible in the near future.

Key words: RPE, stem cells, human pluripotent stem cells, differentiation, cell transplantation

1. INTRODUCTION

Retinal pigment epithelium (RPE) is a highly polarized monolayer of cells located between the neural retina and the choroid (see Figure 1). RPE has several vitally important functions as a part of the blood-retinal barrier and in supporting the neural retina: RPE cells provide nutrients for photoreceptors, phagocytose photoreceptor outer segments, secrete important molecules including pigment epithelium-derived factor (PEDF) and vascular endothelial growth factor (VEGF), absorb stray light, and control regeneration of visual pigments, ion flow and oxidative damage. For a more extensive review of RPE functions and characteristics, article by Strauss is highly recommended (Strauss 2005).

Retinal degenerative diseases, such as age-related macular degeneration (AMD), retinitis pigmentosa, and Stargardt macular dystrophy, affect tens of millions of individuals worldwide. For example in AMD, the macula of the eye gradually degenerates, leading to the loss of central vision, and thus hindering tasks such as face recognition, reading, and driving – important cornerstones for individual independence and quality of life. With a steadily increasing life expectancy, the number of people suffering from AMD worldwide is predicted to increase to almost 200 million by the year 2020 and to over 280 million by 2040 (Wong et al. 2014). There are two types of AMD: dry (atrophic) and wet (neovascular). The dry type accounts for approximately 85% to 90% of all AMD cases. In its advanced form, known as geographic atrophy (GA), dry AMD leads to RPE degeneration and subsequently photoreceptor death in the macula. The underlying cause of dry AMD remains unknown. Wet AMD is caused by abnormal neovascularization from the choriocapillaris beneath the macula. These new blood vessels tend to bleed, leak fluid, and scar, which damages the photoreceptors. Although there is no cure for AMD, repeated injections of anti-vascular drugs into the eye can slow down the neovascularization and progression of early-stage wet AMD (Rosenfeld et al. 2006; Martin et al. 2011; Heier et al. 2012). On the other hand, no effective medical or surgical

treatment is available for dry AMD, although dietary supplements have been suggested to slow its progression (Group 2013). There is clearly a need to develop a therapy that could preserve or repopulate this important RPE cell layer.

2. CELL BASED THERAPY FOR RPE

One of the most promising future treatments for retinal degeneration is cell replacement therapy (Zarbin 2016). The eye in general is a very attractive target for tissue engineering and cell therapy for several reasons. Firstly, the eye offers easy access to well-developed surgical approaches and non-invasive follow-up methods including high resolution optical coherence tomography (OCT) (Adhi and Duker 2013; Zarbin 2016). Secondly, a relatively small number of cells is sufficient for a cell replacement in the eye as compared to many other organs and tissues. Finally, the eye is generally less prone to immune rejection of transplanted cells, although this advantage may be compromised due to disease pathogenesis such as neovascularization of wet AMD (Zhou and Caspi 2010; Stein-Streilein 2013).

The single layer of RPE that lies on Bruch's membrane between the photoreceptor outer segments and the choriocapillaris (Figure 1) is a relatively easy target for cell replacement therapy, compared to highly complex neural retina with functional neural connections. Surgical attempts have been made to replace the RPE at the macula, either by moving the macula to the non-diseased periphery or by grafting new RPE under the macula (Ramsden et al. 2013). These are difficult surgeries to perform and can lead to complications such as unplanned retinal detachment, cataract, and double vision (Stanga et al. 2002). Thus, it is unlikely that these procedures will prove cost effective in combatting the burden of AMD (Ramsden et al. 2013). The earliest attempts of RPE cell transplantation in animal models provided evidence that cell replacement therapy could have potential in treating retinal degeneration. Although the success of these early studies was quite low (Jha and Bharti 2015), they

encouraged further development of the technology. Later, clinical trials were conducted using fetal (Algvere et al. 1994) or post-mortem adult RPE cells (Peyman et al. 1991; Del Priore et al. 2001; Tezel et al. 2007), as well as retina-RPE complex (Radtke et al. 2008). Unfortunately, visual acuity of the patients did not improve in the long term. Among these studies, Binder and colleagues were the first to report autologous RPE transplantation for AMD patients with promising outcomes (Binder et al. 2002). Taken together, these and other early studies not mentioned here provided proof of concept that RPE cell replacement therapy is possible if a viable source of functional RPE cells is established.

As reviewed by da Cruz and colleagues (da Cruz et al. 2007) several cell sources for RPE transplantation have been considered: fetal, autologous, or allogeneic RPE, immortalized RPE cell lines, and stem cells. Among these, the use of fetal tissue is restricted mainly due to poor availability. Autologous RPE cells, on the other hand, may have genetic defects or be functionally impaired due to the disease. Immortalized cell lines likely contain mutations and genetic abnormalities. Thus, the most promising option is either allogeneic RPE cells or RPE cells differentiated from stem cells. Adult human RPE cells isolated from donated eyes can be activated *in vitro* into a stem cell state (RPE stem cells) which are polarized, express RPE markers and have the key physiological properties of native RPE cells making them a candidate for future cell replacement therapy (Salero et al. 2012; Blenkinsop et al. 2015). It is estimated that the macula harbors around 60,000 RPE cells that potentially need replacing. To achieve this, it is necessary to develop technologies to expand RPE stem cells *in vitro*, or to use their paracrine effects to trigger rejuvenation of native RPE *in vivo* (Zhao et al. 2017). Further preclinical studies are ongoing to develop adult RPE stem cell transplantations towards clinical trials (Davis et al. 2016). In addition to the adult RPE stem cells, other stem cell types have been investigated as a source of RPE. Notably, human pluripotent stem cells (hPSC), with their excellent developmental and replicative capacity, can potentially provide an unlimited supply of RPE cells needed to treat the millions of patients suffering from retinal degeneration.

Human embryonic stem cells (hESCs) are usually isolated from surplus embryos of poor quality 4-6 days after *in vitro* fertilization (Skottman 2010). Due to ethical issues and relatively low availability of these cells, as well as their immunogenic properties, the discovery of the possibility to reprogram human somatic cells to behave like hESCs offered even more exciting opportunities for regenerative medicine (Takahashi et al. 2007). After the discovery of human induced pluripotent stem cells (hiPSC) in 2007, numerous non-integrating and non-viral reprogramming methods have been developed using various cell sources including skin fibroblasts, hair follicles, muscle, peripheral blood lymphocytes and urine (Brandl et al. 2015). In many respects, hiPSCs resemble hESCs, although epigenetic and genetic abnormalities in the hiPSC lines have been reported (Huang et al. 2014). This has raised the issue whether epigenetic marks from the cell source may persist in the reprogrammed hiPSCs. This issue has been under critical evaluation as genomic instability in general is recognized as an important hurdle in the expanding field of stem cell-based therapies. According to the current knowledge, the epigenetic differences observed in some hiPSC lines compared to hESC lines seem to be caused mainly by the reprogramming method and diminish during passaging (Chin et al. 2009; Polo et al. 2010; Nishino et al. 2011). Further studies are needed to set a threshold for the acceptable level and genomic location of potential epigenetic and genetic changes in the stem cell product manufactured for clinical applications.

3. DIFFERENTIATION OF RPE FROM HUMAN PLURIPOTENT STEM CELLS

So how can functional RPE cells be obtained from hPSC? During mammalian development, RPE and neural retina both develop from the optic neuroepithelium and share the same progenitors. The neuroepithelium near the anterior part of the neural tube evaginates laterally to form the optic vesicles. Invagination of the distal part of the optic vesicle leads to the formation of the optic cup. By the sixth

or seventh week of gestation, the optic cup has differentiated into two epithelial layers. The distal layer then differentiates into the neural retina and the proximal layer develops into the RPE (Fuhrmann et al. 2014). Since the pioneering work by Sasai and co-workers, hPSC-derived eye organoids mimicking the early retinal developmental steps have been extensively used for modeling eye development *in vitro* (Eiraku et al. 2011). Similarly, *in vitro* differentiation of RPE cells from hPSCs follows the same developmental steps. In 2004, Klimanskaya and co-workers were the first to report successful differentiation of RPE cells from hESCs (Klimanskaya et al. 2004). Many research groups later demonstrated the same also with hiPSCs (Carr et al. 2009b; Hiramani et al. 2009; Buchholz et al. 2009). Since then, numerous research groups have developed methods for obtaining RPE cells from hPSCs, with varying efficiencies. Recent review describe the various differentiation methods in more detail (Song and Bharti 2016), only the general approaches are presented here.

Human PSCs are typically cultured as colonies either on top of a layer of fibroblast feeder cells (mouse embryonic or human foreskin), or without feeder cells (feeder-free) on specific culture substrate in the presence of basic fibroblast growth factor (bFGF) (Skottman and Hovatta 2006; Unger et al. 2008; Crocco et al. 2013). There are two main approaches to initiate RPE differentiation: spontaneous RPE differentiation upon removal of bFGF from the culture medium or directed differentiation using growth factors, inhibitors and/or small molecules. In methods relying on spontaneous differentiation, RPE can be obtained through adherent over growth of hPSC cultures e.g. (Klimanskaya et al. 2004; Lund et al. 2006; Vugler et al. 2008), or by growing embryoid body-like structures in suspension and later plating them down as adherent cultures e.g. (Meyer et al. 2009; Vaajasaari et al. 2011). The more directed differentiation approaches attempt to replicate embryonic development by adding specific growth factors, inhibitors, or small molecules at appropriate time points (Idelson et al. 2009; Zhu et al. 2011; Buchholz et al. 2013; Rowland et al. 2013; Maruotti et

al. 2015). As an example, we have established our own feeder cell-free culture and differentiation method for hPSC-RPE using both of these approaches (Hongisto et al. 2017).

Figure 2 summarise the differentiation and characterisation approach of RPE cells. In general, depending on the method and cell lines used, pigmented foci usually appear in cultures within one to four weeks (see Figure 2A). The pigmented areas are mechanically or enzymatically separated for RPE enrichment. Once separated, pigmented hPSC-RPE cells are seeded on substratum which resembles Bruch's membrane or contains its extracellular matrix (ECM) components such as collagens and laminins (Sorkio et al. 2014). After seeding, hPSC-RPE cells first lose their cobblestone morphology and pigmentation, but regain these characteristics within a few weeks (Abu Khamidakh et al. 2016) (also highly depending on culture conditions). However, if consecutively passaged, hPSC-RPE cells gradually lose their ability to re-establish RPE features (Singh et al. 2013). This is problematic especially if multiple cell doublings are needed to obtain large enough quantities of pure RPE cells. Rho kinase (ROCK) inhibition during passaging may help extend hPSC-RPE passage (Croze et al. 2016), although it remains to be seen how this affects cell functionality and genetic stability. It is also well acknowledged that hPSC-RPE cells require an additional culture period of several weeks—preferably on permeable cell culture inserts—until they mature to a fully polarized and pigmented monolayer (Juuti-Uusitalo et al. 2013a, 2015; Hongisto et al. 2017). Furthermore, culture conditions such as the type of substratum and ECM protein coating highly affect hPSC-RPE maturation (Sorkio et al. 2014).

Different hPSC lines may respond very differently to exogenous signals, making it challenging to develop a universal RPE differentiation protocol. It is unlikely that 100% RPE differentiation efficiency will ever be achieved. Thus, further improvements are needed to increase the yield and purity of the RPE cultures in order to obtain sufficient amounts of mature cells with RPE

characteristics for safe therapeutic use. Besides establishing more efficient hPSC-RPE differentiation methods, another strategy to improve the purity of RPE populations is to sort cells based on RPE specific marker expression (Choudhary and Whiting 2016). As safety is a primary consideration for any clinical use of hPSC-RPE and due to the high risk of tumorigenicity, methods to detect any trace of pluripotent cells among the differentiated cells are critically important (Kuroda et al. 2012; Kanemura et al. 2014; Kawamata et al. 2015). Another safety concern is the use of animal-derived material such as fetal bovine serum (FBS) and mouse feeder cells. These components are often used in establishment, culture and differentiation of hPSCs, and may transfer non-human pathogens to the patient and cause immune reactions (Martin et al. 2005; Sakamoto et al. 2007). Finally, cell therapy applications require defined and reproducible conditions in accordance with Good Manufacturing Practice (GMP) during derivation and maintenance of hPSC lines and RPE differentiation (Unger et al. 2008; Vaajasaari et al. 2011). Overall, the variations in differentiation and culture methods influence hPSC-RPE characteristics, which is why critical consideration and planning is needed when aiming for clinical applications. But first, does hPSC-RPE resemble and behave like native human RPE?

4. CHARACTERIZATION OF STEM CELL -DERIVED RPE CELLS

As mentioned earlier, RPE cells have many vitally important characteristics and functions (Strauss 2005). Thus, it is necessary to verify that hPSC-RPE cells possess these characteristics (See Figure 2B). This is critically important for clinical use, but also for non-clinical applications where hPSC-RPE cells serve as a model of native human RPE. Since the very early studies, putative hPSC-RPE cells were proven to share many characteristics with authentic human RPE: they express RPE specific genes and proteins (e.g. Bestrophin, CRALBP, Na⁺/K⁺ATPase, MERTK, Zonula Occludens-1 and Claudin-19), have pigmented cobblestone-like morphology, form tight and highly polarized RPE with high transepithelial electrical resistance (TEER), phagocytose isolated photoreceptor outer segments,

and secrete growth factors such as PEDF (Klimanskaya et al. 2004; Carr et al. 2009a; Lu et al. 2009; Buchholz et al. 2009; Liao et al. 2010; Vaajasaari et al. 2011; Kamao et al. 2014). Moreover, hPSC-RPE cells express many important transporters (Kokkinaki et al. 2011; Juuti-Uusitalo et al. 2012) and aquaporin water channels (Juuti-Uusitalo et al. 2013a), and possess other physiologically relevant functions (Miyagishima et al. 2016). For instance, AMD patient-derived hiPSC-RPE cells have decreased antioxidative defence compared to healthy hiPSC-RPE cells, providing proof of concept that their stress response properties are similar to that of native RPE (Chang et al. 2014). RPE cells derived from hiPSCs are in many respects very similar to hESC-RPE cells (Juuti-Uusitalo et al. 2013b; Hongisto et al. 2017). Furthermore, a recent comparison of hiPSC lines derived from different somatic cells suggests that hiPSC-RPE functions are more significantly affected by the genetic background of different donors, than the epigenetic “memory” associated with the donor tissue (Miyagishima et al. 2016). In addition, there is always heterogeneity among hPSC-RPE cultures and cell characteristics and functionality may vary. Thus, it is important to define RPE identity (Bharti et al. 2011, 2014) and improve quantitative methods to identify different maturation stages of hPSC-RPE cells. There is ongoing discussion regarding hPSC-RPE characteristics and the various criteria these cells should fulfil in order to be considered authentic RPE cells (Bharti et al. 2011). For example, Buccholz and co-workers have suggested that the systematic characterization panel of hPSC-RPE should include at least gene and protein analyses, quantitative phagocytosis, TEER measurement, growth factor secretion analysis, retinoid metabolism assay, and functionality in an animal model (Buchholz et al. 2009). To conclude, there is an extensive and ever increasing amount of studies supporting the current knowledge that hPSCs are a promising source of functional RPE cells. The main down-stream applications of these cells are for modelling native RPE for drug testing, for modelling retinal diseases (Yvon et al. 2015), and for RPE cell replacement therapies—the focus of the following subchapters.

5. HUMAN PSC-RPE TRANSPLANTATION STUDIES IN ANIMAL MODELS

The efficacy of hPSC-RPE cell therapy has been extensively studied in different animal models and only some of these are mentioned here as examples. Although large-eyed animal models are preferred especially for the development of subretinal transplantation techniques (Stanzel et al. 2012; Brant Fernandes et al. 2016; Thielges et al. 2016; Al-Nawaiseh et al. 2016), the Royal College of Surgeons (RCS) rat remains a widely used animal model. Its RPE is unable to phagocytose photoreceptor outer segments, and therefore photoreceptors degenerate over a period of 3 months after birth (Dowling and Sidman 1962; Mullen and LaVail 1976; Edwards and Szamier 1977). The Food and Drug Administration (FDA) recommends the use of this animal model to demonstrate safety and efficacy of hPSC-RPE (Bharti et al. 2014). The hPSC-RPE transplantation studies follow one of two approaches: injection of a single-cell suspension into the subretinal space, or transplantation of an hPSC-RPE sheet with or without supportive biomaterial matrix (See Figure 1). Both of these approaches have their own pros and cons which are briefly discussed next.

5.1 Injection-based transplantation

In the first RPE transplantation studies in 2004, primate ESC-derived RPE injected into the subretinal space of RCS rats helped recover retinal function (Haruta et al. 2004). It was later demonstrated that although single cell suspension injected hPSC-RPE cells survive and improve visual acuity in RCS rats, they rarely form tight epithelia after transplantation and gradually die within 10–15 weeks and cell survival for up to 20–30 weeks seems to be an exception (Lund et al. 2006; Vugler et al. 2008; Carr et al. 2009b; Idelson et al. 2009; Lu et al. 2009; Krohne et al. 2012). Besides RCS rats, hPSC-RPE cells have been studied in monkeys and nude rats with a similar outcome (Diniz et al. 2013; Kamao et al. 2017). The improvement of visual function has been assessed using electroretinography (ERG) and even using behavioral assays that measure eye or body movements in response to light

(optokinetic responses) (Carr et al. 2009b; Idelson et al. 2009). The temporary improvements in visual acuity of the RCS rat are thought to be more due to trophic factors (Shi et al. 2008) secreted by the transplanted cells, or macrophages that might help to phagocytose photoreceptor outer segments (Carr et al. 2009a). It has been suggested that failure to maintain a long-term improvement may be due to the impaired survival of hPSC-RPE on diseased Bruch's membrane (Sugino et al. 2011a, c; Petrusreurer et al. 2017). However, injection-based transplantation is fast and technically less challenging than RPE sheet transplantation, which is why it was selected for the first clinical studies in human patients.

5.2 RPE sheet transplantation

RPE cells are sensitive to local extracellular substrates for anchoring and survival (Tezel and Del Priore 1997), so transplantation of a pre-formed, oriented, polarized monolayer with tight junctions could enhance cell viability and integration into the retina (Zhu et al. 2011; Diniz et al. 2013). Moreover, intact RPE monolayers have a higher resistance for oxidative stress and thus could survive better in diseased retina (Zhu et al. 2011). Finally, the required amount of cells for sheet transplantation is much lower than for subretinal injections (Diniz et al. 2013). Disadvantages of the transvitreal sheet transplantation are its invasiveness and demands of surgical procedure, although surgical techniques and specialized tools have been developed to ease RPE sheet transplantation into the back of the eye (Stanzel et al. 2012; Al-Nawaiseh et al. 2016).

Retinal degeneration often involves Bruch's membrane – the dynamic, 2-4.7 μm thick, pentalaminar structure which mainly consists of collagens, elastins, laminins, and fibronectin. Its thickness and permeability varies with age, pathological stage, and retinal location (Ramrattan et al. 1994; Booij et al. 2010). The aged and thickened submacular Bruch's membrane does not support long-term survival

and differentiation of transplanted RPE (Gullapalli et al. 2005; Sugino et al. 2011b). Consequently, it could be beneficial to transplant hPSC-RPE sheet with a supportive biomaterial scaffold to simultaneously substitute RPE and Bruch's membrane function. In order to best mimic the properties of Bruch's membrane, biomaterial substrates for production and transplantation of hPSC-RPE cells should meet several requirements. First, the scaffold material should support formation of tight hPSC-RPE with proper apical-basal polarization as well as native RPE characteristics. Second, the substrate should be biocompatible, thin enough to fit the subretinal space, with mechanical properties suitable for handling of the cell sheet. Third, and most important, the material needs to enable integration of transplanted cells to the retina. Finally, permeability to fluids and biomolecules is a definite prerequisite for substrates to replace the lost functional role of the damaged Bruch's membrane as a semipermeable barrier (Hynes and Lavik 2010; Binder 2011; Pennington and Clegg 2016; Sorkio et al. 2017).

Many research groups are focusing on finding an optimal scaffold for RPE transplantation. Decellularized natural scaffolds, such as Bruch's membrane, amniotic membrane, and anterior lens capsule, have been previously suggested as substrates in retinal transplantations (Nicolini et al. 2000; Akrami et al. 2011; Kiilgaard et al. 2012). These natural scaffolds provide a significant advantage in retaining the complex structure and molecular hierarchy of the ECM while possessing tissue-specific micro- and nanotopography (Hynes and Lavik 2010; Walters and Gentleman 2015). In addition, natural polymers such as collagen, alginate, and fibroin, provide biocompatible sources of polymers for retinal tissue engineering. Natural polymers also closely mimic the native ECM and possess innate biological activity (Hynes and Lavik 2010; Rahmany and Van Dyke 2013). Still, biomaterials of natural origin have several disadvantages including poor mechanical properties, batch to batch variation, as well as concerns with immunogenicity, toxic by-products of biodegradation, and pathogen transfer.

Synthetic polymers have multiple attractive characteristics including controlled chemical and physical structure, predictable properties, mechanical durability, high degree of processing flexibility, and high reproducibility in commercial-scale manufacturing processes (Hynes and Lavik 2010). The most commonly used synthetic polymers are poly- α -hydroxy-acid-based polymers such as poly(L-lactide) (PLLA), poly(lactide-co-glycolide) (PLGA), poly(ϵ -caprolactone) (PCL), and combinations of these materials as co-polymers (e.g. PLCL) (Lee et al. 2008; Hynes and Lavik 2010; Sorkio et al. 2015a). Even though synthetic polymers overcome the common drawbacks associated with natural polymers, they tend to be hydrophobic and lack cell binding ligands on the scaffold surface, which results in poor cell attachment without additional surface modifications (Sorkio et al. 2015a). Many synthetic biomaterial substrates with distinct architecture have been investigated as potential substrates for RPE (Sorkio et al. 2015b; Ilmarinen et al. 2015; Pennington and Clegg 2016; Calejo et al. 2016). Finally, hybrid materials incorporate the beneficial aspects of both biologically active natural polymers and structurally flexible synthetic polymers such as combination of silk fibroin, gelatin and PCL (Wang et al. 1999). Hybrid biomaterials show promise as potential Bruch's membrane mimicking substrates for RPE (Warnke et al. 2013; Xiang et al. 2014).

Most biomaterial studies use either primary RPE cells or immortalized cell lines, and studies of hPSC-RPE cell-biomaterial interactions have only recently gained popularity e.g. (Kamao et al. 2014; Brant Fernandes et al. 2016). The most disputed property of hPSC-RPE scaffold material is its biostability – are biodegradable scaffolds preferable over biologically inert/ non-degradable materials? A biodegradable membrane would provide a temporary support for hPSC-RPE, until the cells remodel and replace it with new ECM layers. A biostable membrane would provide permanent support for the cells and at the same time improve integration of the transplant by providing better permeability, which is likely to be critical for retinal health (Kamao et al. 2014; Stanzel et al. 2014). For instance, Parylene C (poly(para-xylene)) is a biostable and chemically inert polymer. When combined with Matrigel™ or human vitronectin, Parylene C was shown to support hPSC-RPE growth and

functionality both *in vitro* and *in vivo* (Koss et al. 2016). Our group has studied the performance of biologically inert polyimide (PI) for hPSC-RPE culture (Subrizi et al. 2012) and transplantation (Ilmarinen et al. 2015). These approaches aim to overcome the disorganized fashion in which RPE cells adhere to Bruch's membrane when injected as a suspension. The plastic polymer is also designed to act as a replacement for the aged and thickened Bruch's membrane, and provides an anchor for the cells while aiding in surgical delivery (Ramsden et al. 2013). It remains as an important target for future preclinical and clinical studies to demonstrate whether the best efficacy for hPSC-RPE transplantation is achieved with the less invasive injection method or with the surgically challenging sheet transplantation. Alternatively, perhaps the transplantation method should be chosen based on individual patient and disease status. Finally, further clinical studies will need to demonstrate whether improved retinal function translates into improved cortical representation of images—an outcome which has been observed in humans after transplantation of adult RPE (da Cruz et al. 2007).

6. CLINICAL TRIALS WITH HPSC-RPE CELLS

In 2012, eight years after the first report of successful hESC-RPE differentiation, the first Phase I/II clinical trial using these cells was reported by Schwartz and co-workers, with indication of a good safety profile for patients with Stargardt disease and advanced dry AMD (Schwartz et al. 2012). In the three-year follow-up study with 18 participants, hESC-RPE cells were administered as a subretinal injection of three dosage cohorts (50,000 cells, 100,000 cells and 150,000 cells). In addition to the safety of the treatment, the authors also demonstrated improved vision in four out of nine AMD patients. Interestingly, only few, if any, pigmented hESC-RPE cells survived in the direct area of GA lesions. Instead, transplanted cells were detected in areas adjacent to the lesions, where they were deposited onto native RPE (Schwartz et al. 2015). A Phase II study with more patients to assess efficacy is expected to report results imminently (Zhao et al. 2017). Other clinical trials with hPSC-

RPE injections are ongoing in several countries including Israel, China, and Korea (for details see recent review (Zhao et al. 2017) and <https://clinicaltrials.gov>). It remains to be seen if long-term survival and function of subretinally injected cells is achieved.

Unlike hESCs, hiPSCs can be obtained from the patients' own somatic cells, offering the potential for immune-compatibility. To date, one AMD patient has been treated with autologous hiPSC-RPE cells manufactured and transplanted without an artificial scaffold (Mandai et al. 2017). RPE cells grown on a collagen gel were enzymatically lifted and transplanted as a sheet into the subretinal space of a patient with advanced wet AMD in Japan. It was reported that the patient did not experience any serious side effects and maintained visual acuity one year after surgery. The patient did not receive immunosuppressants and showed no signs of rejection. A second patient was recruited to the study, but was put on hold due to genetic mutations in hiPSC-RPE cells. (Garber 2015; Mandai et al. 2017) Since then, the approach was modified towards using allogeneic hiPSCs and the clinical trial has resumed (Zhao et al. 2017)

In a clinical trial carried out by The London Project to Cure Blindness in collaboration with Pfizer, two patients with wet AMD were treated with hESC-RPE monolayer immobilized on a polyester membrane (Coffey 2017; da Cruz et al. 2018). This is the first study demonstrating successful delivery and survival of hESC-RPE patch with a visual acuity gain of two patients treated. Similarly, a clinical trial led by Regenerative Patch Technologies (USA) is aiming to treat GA by transplanting hESC-RPE sheet on parylene C membrane and have very recently published first positive results with 4 out of 5 patients treated (Kashani et al. 2018). In both of these studies a non-degradable biomaterial with permanent support is used. In contrast, in a clinical trial planned by NEI/NIH, Bharti and co-workers aim to use a biodegradable matrix which will gradually dissolve after successful delivery of the hPSC-RPE cell sheet (Song and Bharti 2016). Perhaps the final outcomes of these trials will demonstrate what type of biomaterial substrates is better suited for hPSC-RPE delivery—biostable or

biodegradable. Overall, it is very encouraging that studies using both cell delivery methods, and both hPSC types, appear to be safe in initial clinical studies.

7. FUTURE PERSPECTIVES FOR RPE CELL THERAPY

Although many clinical trials are ongoing, there are several open questions that need to be addressed and properly answered before safe and effective hPSC based cell therapies are widely available. One of the important and unanswered questions is whether to use hESC or hiPSC for cell replacement therapies. Although considered as functionally equivalent to hESCs, hiPSCs have been shown to harbor subtle differences in gene expression and DNA methylation (Doi et al. 2009). Furthermore, there have been reports of point mutations and copy number variation in hiPSCs, which raises possible safety issues (Howden et al. 2011; Mandai et al. 2017).

The use of hPSC-RPE in clinical application faces multiple challenges, including manufacturing and characterization of clinical grade cells. The precise list of functional properties absolutely required prior to transplantation, and properties that the cells may or may not acquire once they are correctly integrated into the host tissue, is still missing (Bharti et al. 2011).

Potential tumorigenicity of transplanted cells is a challenge that needs method development and increased understanding to guarantee safety and efficacy of transplanted cells. The pluripotent nature of hPSCs also raises the concern that if any undifferentiated hPSCs were left in the final clinical product, they could increase the risk of tumor or teratoma formation after transplantation. Another challenge is the immune-acceptance of transplanted cells. Although the subretinal space is relatively immune-privileged, damaged blood–retinal barrier, leaky blood vessels, and activated microglia may be present in diseased retina or induced by surgery. This can compromise the immune-privilege and cause cell rejection. Immunogenicity of allogeneic hPSC-RPE cells is therefore an issue (Sugita et al. 2016a, b). Autologous hiPSC-RPE cells offer minimal risk of cell rejection, but would not be a cost

effective strategy on a large scale. Thus a thorough understanding of the immunogenicity of hPSC-RPE and the optimal immunosuppression regime is essential for future clinical applications. In addition, international cell banking initiatives covering different human leukocyte antigen (HLA) types or universal hPSC lines with standardized cell banking and production methods are needed (Gornalusse et al. 2017). Otherwise there is a well-recognized risk that these treatments remain unaffordable for the majority of the patients.

Overall, multifactorial diseases such as AMD need further understanding of the disease pathogenesis, and most likely efficient treatment will require multidisciplinary approaches and personalized medicine. In addition, combination therapy of cell replacement, gene correction, supportive biomaterials and pharmaceuticals may be needed for certain diseases. First, as an example, strategies are being developed to genetically correct hiPSCs prior to differentiation and autologous transplantation (Meyer et al. 2011). Second, in addition to RPE and photoreceptor degeneration, Bruch's membrane alterations including thickening and accumulation of drusen play an important role in AMD pathogenesis. Thus further studies are needed to see if artificial scaffolds can replace Bruch's membrane enabling proper RPE cell attachment in the diseased eye, or if novel methods are developed to improve attachment and polarization of injected RPE cells. Third, the critical cell type in early AMD seems to be RPE, while in more advanced cases, it may be necessary to replace neuronal cells either alone or together with RPE cells. Production and transplantation of photoreceptors or their progenitors has shown some promise, but is challenging due to the requirement for functional integration and synaptic contacts with the host neurons (Singh et al. 2016). Last but not least, combination of effective medication and new therapeutic agents may be needed together with cell-based therapies to improve their efficacy.

In the end, it would be devastating for the whole field of regenerative medicine if any harm was caused to the patients in ongoing and upcoming clinical trials with hPSC-derived cells. Thus it is

important to establish world-wide standards for the proper preclinical and clinical studies to minimize and hopefully avoid poor outcomes (Marks et al. 2017). Several clinical trials are in progress around the world and the results will undoubtedly be exciting, but continued research and collaboration are needed to ensure above all safety, and then success of these ground-breaking approaches.

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Figure legends:

Figure 1. The hPSC-RPE transplantations. The hPSC-RPE cells can be efficiently differentiated and transplanted to the subretinal space between neural retina and choroid. Currently two different transplantation approaches are used A) single-cell suspension injection and B) transplantation of intact RPE cell sheet.

Figure 2. The general hPSC-RPE differentiation and characterisation approach. A) hPSC-RPE differentiation can be divided in induction of differentiation, purification and expansion of pigmented cells, additional replating/passaging and cryopreservation of cells and finally hPSC-RPE maturation and characterisations. Scale bar 100µm. B) hPSC-RPE characterized with expression and localization of Zonula Occludens-1 (ZO1), Na⁺/K⁺ATPase, RPE65, MERTK proteins and phagocytosis of photoreceptor outer segments (OPSIN). Scale bar 10 µm.

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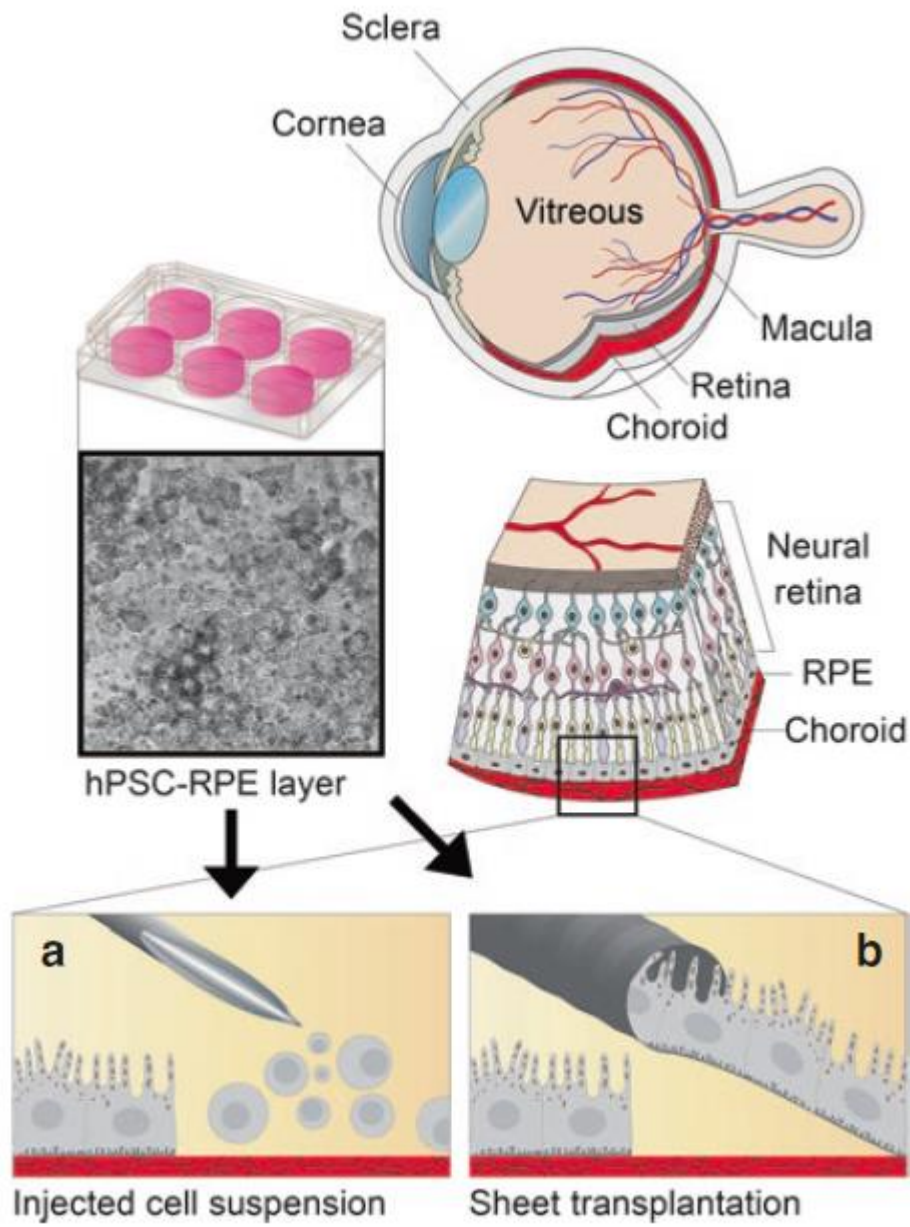


Figure 1.

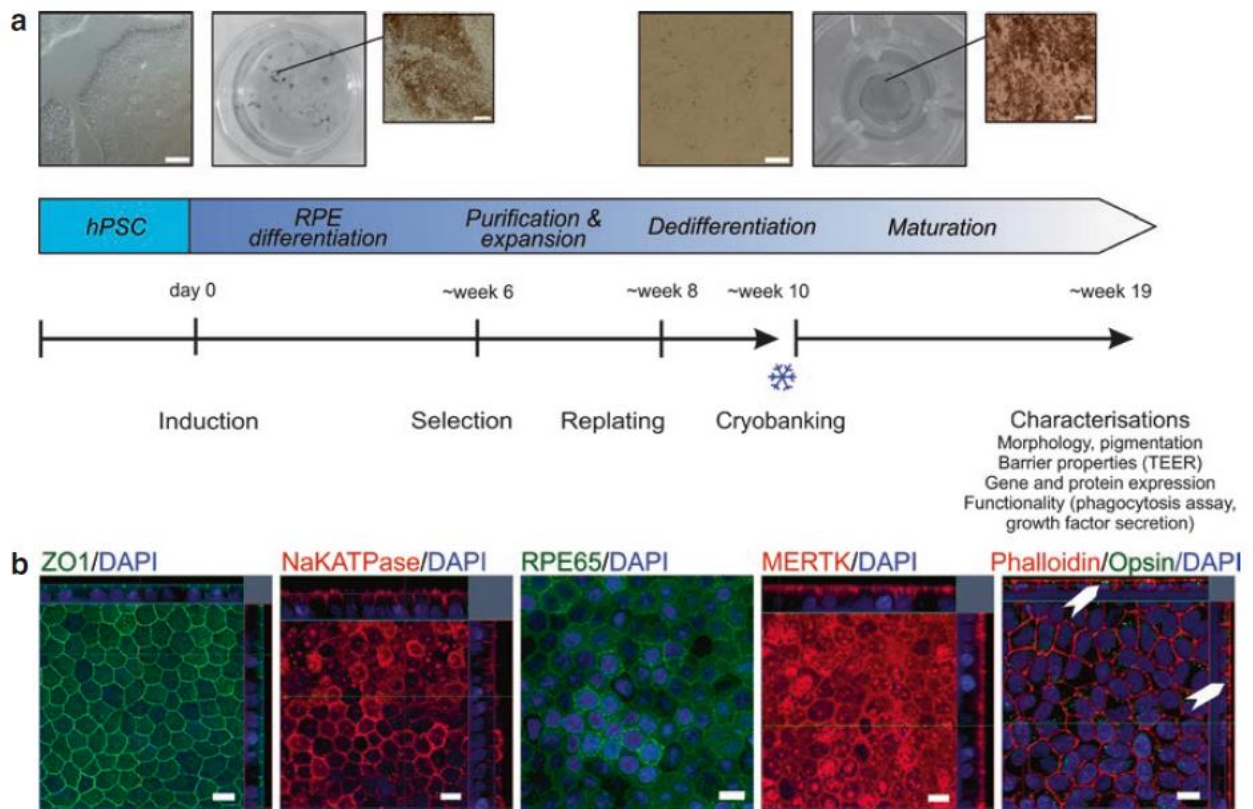


Figure 2.