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Looking into the Future: Using Induced Pluripotent Stem Cells to Build Two and Three Dimensional Ocular Tissue for Cell Therapy and Disease Modeling

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Abstract

Retinal degenerative diseases are the leading cause of irreversible vision loss in developed countries. In many cases the diseases originate in the homeostatic unit in the back of the eye that contains the retina, retinal pigment epithelium (RPE) and the choriocapillaris. RPE is a central and a critical component of this homeostatic unit, maintaining photoreceptor function and survival on the apical side and choriocapillaris health on the basal side. In diseases like age-related macular degeneration (AMD), it is thought that RPE dysfunctions cause disease-initiating events and as the RPE degenerates photoreceptors begin to die and patients start losing vision. Patient-specific induced pluripotent stem (iPS) cell-derived RPE provides direct access to a patient's genetics and allow the possibility of identifying the initiating events of RPE-associated degenerative diseases. Furthermore, iPS cell-derived RPE cells are being tested as a potential cell replacement in disease stages with RPE atrophy. In this article we summarize the recent progress in the field of iPS cell-derived RPE “disease modeling” and cell therapies and also discuss the possibilities of developing a model of the entire homeostatic unit to aid in studying disease processes in the future.

Keywords

retinal degeneration; age related macular degeneration; transplantation; retinal pigment epithelium; induced pluripotent stem cells; tissue engineering; microfluidics; 3D disease models

Introduction

Need for *in vitro* Models of the Ocular Homeostatic Unit

The functional light-sensing unit in the back of the eye consists of a neurosensory retina, the retinal pigment epithelium (RPE), the proteinaceous Bruch's membrane, and the endothelial cells that line the choriocapillaris. Photoreceptors of the retina are the main light-sensing cells of this unit, whereas the RPE along with the structural support from the Bruch's

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membrane, and endothelial cells form the outer blood retina barrier (BRB) for this unit. Together, these cell types are also called the homeostatic unit in the back of the eye (Fig. 1A) (Bharti et al., 2011). The RPE is strategically located in between the neurosensory retinal layer and Bruch's membrane and is critical for maintaining the health and integrity of this entire homeostatic unit (Fig. 1A). The RPE performs several functions that are critical for photoreceptor and choriocapillaris survival and health, including: (1) transport of nutrients such as glucose, O₂, and vitamin A from the choriocapillaris to the photoreceptors that are not in direct contact with any blood supply; (2) phagocytosis of photoreceptor outer segments that have been damaged by photooxidation; (3) maintenance of the visual cycle - as light hits photoreceptors, opsin-bound visual pigment 11-cis retinal is isomerized to all-trans retinal and released from opsin, and then the RPE reisomerizes it back to the functional form 11-cis retinal; (4) maintenance of the chemical composition of the sub-retinal space by regulating the K⁺ concentration to physiological levels of 5 mM and by removing CO₂ from the sub-retinal space produced during photoreceptor respiratory cycle; (5) controlling the volume of the subretinal space and the choroid by transporting water from the sub-retinal space to choriocapillaris; and (6) constitutively secreting cytokines in a polarized fashion towards the retina and the choroid to regulate their development, function, and pathophysiology (Adijanto et al., 2009; Bharti et al., 2011; Li et al., 2009; Li et al., 2011; Maminishkis et al., 2006; Maminishkis and Miller, 2010; Mitchell et al., 2011; Shi et al., 2008; Strauss, 2005). Functional defects in the RPE lead to physiological defects in the entire homeostatic unit and are the hallmark features in several degenerative retinal diseases, both monogenic (e.g. Stargardt and Sorsby's fundus dystrophy) and polygenic (e.g. age-related macular degeneration (AMD) (Ambati and Fowler, 2012; Ambati et al., 2013; Langton et al., 2005; Zhong and Molday, 2010). Discovery and elucidation of early initiating events in these diseases that originate in the RPE could allow development of clinical interventions so that the homeostasis of the entire unit could be rescued. Sorsby's fundus dystrophy and AMD are typical examples of diseases where the primary functional defect originates in RPE cells, but disease processes that follow spread across the entire homeostatic unit.

Sorsby's fundus dystrophy is a rare and genetically dominant disease caused by mutation in a matrix metalloproteinase inhibitor gene *TIMP3* (Weber et al., 1994). The *TIMP3* gene is highly expressed in the RPE and the protein is located on the basal side of RPE in the Bruch's membrane (Strunnikova et al., 2010; Weber et al., 2002). Studies performed in human cell lines and mouse models suggest that mutant TIMP3 protein degrades slower compared to the wild type protein, and likely because of this slower degradation it accumulates in RPE cells and in the Bruch's membrane (Weber et al., 2002 IOVS; Langton et al., 2005). The consequence of mutant TIMP3 accumulation, however, is not clear. One study suggests that mutant protein is weaker at suppressing matrix metalloproteinases (MMPs). Increased MMP activity can enhance angiogenesis in choriocapillaris (Qi et al., 2002). Another study suggests that accumulation of mutant TIMP3 or its reduced activity changes the hydraulic conductivity of Bruch's membrane. This results in a reduced flow of nutrients and oxygen from the choriocapillaris towards the RPE and the photoreceptors (Booij et al., 2010; Langton et al., 2005). Reduced oxygen absorption can cause hypoxia in the back of the eye, increase the secretion of angiogenic factor VEGF, and lead to hemorrhage and leakage in choriocapillaris. Part of the reason why it has not been feasible to

understand the initiating events of such a complex disease is because there are no good human models to study the intersection of RPE, Bruch's membrane, and choriocapillaris.

Similar to Sorsby's fundus dystrophy, AMD affects all the three cell types in the back of the eye. But, unlike Sorsby's fundus dystrophy that affects the whole retina, AMD is more restricted to the center of the eye (the macula). It has two phenotypically distinct advanced stages, the “dry” stage or geographic atrophy (GA) and the “wet” stage or choroidal neovascularization (CNV) (Fig. 1). Clinically GA is defined as a stage with atrophy of RPE cells (Ambati et al., 2013; Zarbin et al., 2014). RPE cell death in GA is tightly coupled with accumulated oxidative and metabolic insult ensued by photoreceptors and is associated with a corresponding loss of choroidal blood vessels (Fig. 1B,C) (Ambati et al., 2013; Arjamaa et al., 2009; Kinnunen et al., 2012). In comparison, CNV is associated with abnormal growth of choroidal blood vessels (Fig. 1D). At the cellular level, it is widely accepted that increased basal VEGF secretion by RPE induces CNV. This increase in VEGF secretion is likely caused by intracellular inflammation and hypoxia in the RPE, likely through the activation of HIF-1 alpha, NRF2, and NF-kB pathways (Arjamaa et al., 2009; Wang et al., 2014). There are no good animal models that can recapitulate all the clinical hallmarks of an early to advanced AMD stages. The iPS cell technology has recently provided a simple two-dimensional model of patient-derived RPE to study specific stages of AMD pathogenesis (Yang et al., 2014). It is hoped that in the future this technology can be extended to develop complex 3-dimensional models of the back of the eye.

Potential Uses of iPS Cell Derived Ocular Tissues

Achieving complex 3D models of the RPE as part of the homeostatic unit could allow the discovery of early initiating events in diseases such as AMD and Sorsby's fundus dystrophy. Identification of potential drugs that target these early events help restore the homeostasis of the entire unit. In cases where initiating events cannot be controlled and disease has resulted in atrophy of ocular tissue, replacing the damaged tissue with a “new” healthy tissue would be ideal, and is an area of focus by several ongoing trials (Bharti et al., 2014). Tissue replacement efforts will depend on the extent of degeneration of the host tissue. In cases where only RPE is atrophied, there will be a need to replace it with healthy RPE, for example in cases of early “dry” or “wet” form of AMD (Schwartz et al., 2015); for a detailed review see (Carr et al., 2013). However, in cases where other parts of the homeostatic unit are also damaged, there will be a need to replace some or all components of the unit (e.g. RPE, photoreceptors, and choriocapillaris replacement in late stages of “dry” AMD or in certain types of retinitis pigmentosa) (Wright et al., 2014). Currently, iPS cell technology holds potential for developing both 2D and 3D cell replacement therapies for degenerative diseases of the eye. Multiple ongoing efforts have focused on the 2D models involving only the RPE (Bharti et al., 2014). Some preliminary ongoing work is also addressing the need to develop complex 3D models as potential tissue replacement therapies (Phillips et al., 2012; Zhong et al., 2014).

Thus, current and ongoing work illustrates the need for consideration of the RPE as a critical member of the homeostatic unit, and the need for effective modeling of complex degenerative eye diseases. Here, we will summarize the recent progress in the field of retinal

degenerative disease modeling using the iPS cell technology with a focus on RPE. We will also discuss ongoing and potential future applications of iPS cell-derived RPE as a cell therapy for retinal degenerative diseases. Finally, we will provide insight into future applications of iPS cell technology combined with tissue engineering to develop complex 3-dimensional disease models for retinal degenerative diseases and for cell replacement therapies.

Progress and Challenges

Differentiation of Pluripotent Cells into Functional RPE Monolayer

A critical first step in the development of *in vitro* human disease models and cell therapy is the ability to generate sufficient quantities of functionally validated human “diseased” and healthy RPE cells. Two fundamentally different approaches have been used to differentiate retinal progenitor and RPE/photoreceptor cells from human pluripotent ES and iPS cells (Table 1). One utilizes the spontaneous differentiation ability of pluripotent stem cells (Carr et al., 2009a; Haruta et al., 2004; Kawasaki et al., 2002; Klimanskaya et al., 2004; Liao et al., 2010; Lund et al., 2006; Vugler et al., 2007; Vugler et al., 2008). In this method, adherent cultures of ES/iPS cells are differentiated by basic fibroblast growth factor (bFGF) withdrawal and in several weeks pigmented RPE patches appear sporadically in culture dishes. These patches can be manually or enzymatically isolated to obtain almost pure cultures of RPE cells. However, because of the low differentiation efficiency, multiple cell doublings are required to obtain large enough quantities of pure RPE cells. This is a concern, because epithelial cells under repeated passaging conditions tend to lose their epithelial character and undergo a process of epithelial to mesenchymal transition (EMT) (Singh et al., 2013a). Recent work suggests that the addition of an inhibitor of Rho-associated kinase (Rock) Y-27632 can inhibit such EMT processes and help maintain the epithelial character of stem cell-derived RPE (Croze et al., 2014). However, the epigenetic stability of serially expanded stem cell-derived RPE cells with or without Y-27632 is not known and more work is needed to analyze their functionality. In any case, these early protocols provided hope that large number of patient-specific RPE cells can be produced for *in vitro* analysis and for developing cell-based therapies.

The other approach used to differentiate ES or iPS cells into photoreceptors, retinal neurons, or RPE utilizes knowledge of the developmental biology of the eye. It requires step-wise differentiation of pluripotent stem cells into multi-potential neuroectoderm cells, then differentiation of neuroectoderm cells into eye-field progenitors that can be coaxed into either retinal neurons, photoreceptors, or RPE cells by addition of selective growth factors (Buchholz et al., 2013; Carr et al., 2013; Hiram et al., 2009; Idelson et al., 2009; Lamba et al., 2006; Meyer et al., 2009; Osakada et al., 2008; Osakada et al., 2009; Reh et al., 2010; Zhou et al., 2011; Bharti et al., 2012). This protocol uses the embryoid body method to mimic early embryonic development. Embryoid bodies, in principle, should generate cells of all three germ layers – ectoderm, endoderm, and mesoderm. But, inhibition of TGF and BMP (dual-SMAD inhibition), and canonical WNT pathways, and the activation of IGF and FGF pathways at embryoid body stage successfully and selectively induces neuroectoderm with eye-field characteristics within a few days of culture (Adijanto et al., 2009; Buchholz et

al., 2013; Chambers et al., 2009; Idelson et al., 2009; Leach et al., 2015; Meyer et al., 2009; Osakada et al., 2008; Osakada et al., 2009; Reh et al., 2010). These cells express high-levels of several eye-field transcription factors like retinal homeobox (RX), paired-homeodomain transcription factor PAX6, SIX homeobox 3 (SIX3) and SIX6, and LHX2 (Lamba et al., 2006; Meyer et al., 2009). Eye-field cells go on to differentiate into optic vesicle-like cells by approximately 3-4 weeks of culture that coexpress microphthalmia-associated transcription factor (MITF) and PAX6. These cells will spontaneously differentiate into either RPE or retinal progenitors (Meyer et al., 2009; Phillips et al., 2012). PAX6- and MITF-coexpressing progenitor cells can also be efficiently differentiated into the photoreceptor lineage by addition of retinoic acid, SHH, Notch, and Taurine (McUsic et al., 2012; Meyer et al., 2009; Osakada et al., 2009); into retinal neurons by using canonical WNT and TGF agonists (Lamba et al., 2006); and into RPE lineage by RPE-inducing TGF-signals (Bharti et al., 2012; Carr et al., 2013; Idelson et al., 2009; Leach et al., 2015; Meyer et al., 2009; Osakada et al., 2008; Osakada et al., 2009; Reh et al., 2010). Table 1 provides a list of publications that have developed various protocols for ES/iPS cell-to-photoreceptor or RPE differentiation. As compared to spontaneous differentiation, step-wise developmentally guided differentiation methods are certainly more efficient in generating RPE cells. RPE differentiation efficiency as high as 70% has been reported using these protocols (Idelson et al., 2009; Klimanskaya et al., 2004; Osakada et al., 2008). This allows generation of a large quantity of RPE cells without additional cell doublings that could compromise the functionality of cells. Furthermore, use of these protocols has significantly increased the reproducibility of RPE/photoreceptor differentiation when using iPS cell lines derived from different patients or tissue sources.

Authentication of Pluripotent Stem Cell-derived RPE

It is critical that any cell type derived from pluripotent stem cells is fully authenticated before they are used for any potential clinical applications. Fully authenticated cells will likely provide more effective disease models, drugs screening tools, and cell-based therapies. We have recently used a combination of molecular, morphological, and functional assays to authenticate RPE derived from human iPS cells (Ferrer et al., 2014). We used two different functional assays: in one case, we determined baseline cytoplasmic calcium concentration in cells and tested the ability of purinergic membrane receptors to respond to external stimuli and activate endoplasmic reticulum calcium store to change cytoplasmic calcium concentration; in the other case, we tested the ability of apical membrane potassium channels and purinergic receptors to respond to external stimuli and change membrane polarization properties across the entire monolayer of cells. These monolayer properties depend on several key structural and functional features, for example: 1) the formation of appropriate tight junction complexes between neighboring RPE cells that determine the key electrical and mechanical properties of the monolayer; 2) the appropriate apical-basal polarization of the cells; and 3) the functional intactness of several signaling pathways that connect events at the apical membrane to the cytoplasm and to the basal membrane of the cells. We demonstrated that iPS cell-derived RPE monolayer are electrically intact; have a transepithelial resistance $>150 \Omega \cdot \text{cm}^2$; are appropriately polarized with a transepithelial potential of $\sim 2.5 \text{ mV}$ (basal membrane more positive); are able to hyperpolarize in response to changes in extracellular potassium concentration and depolarize in response to

extracellular ATP by mobilizing their intracellular calcium stores. Our work suggests that stringent functional criteria can indeed be used to authenticate iPS cell-derived RPE and such criteria provide a deeper insight into the quality of cells before they are used for translational applications.

Two-dimensional Models of Retinal Degenerative Diseases

The ability to generate patient-specific iPS cells provides a direct access to patient's genetics and allows the possibility of discovering causative links between genotype, cellular endophenotype, and the clinical phenotype. This also allows the possibility of discovering disease-initiating molecular pathways that can provide more effective targets for therapeutic intervention. It is, however, thought that monogenic degenerative diseases are relatively easier to reproduce using this “disease-in-a-dish” approach as compared to age-related and polygenic diseases where environmental influence may change the disease course (Inoue et al., 2014). This is because patient and tissue-specific environmental factors are not easy to replicate *in vitro* (Inoue et al., 2014). Here, we will discuss examples of one monogenic and one polygenic retinal degenerative disease modeled *in vitro* using patient-specific iPS cells.

The first major attempt towards RPE-associated “disease modeling” focused on a monogenic disease called BEST disease (Blodi and Stone, 1990; Singh et al., 2013b). BEST is an autosomal dominant form of macular dystrophy of the RPE and is caused by point mutations in a membrane protein-coding gene *BESTROPHIN-1* or *BEST1* (Boon et al., 2009; Petrukhin et al., 1998). The physiological role of *BEST1* in human RPE is not fully understood, but it is thought to be a calcium-activated chloride channel likely localized on the endoplasmic reticulum and/or plasma membrane (Marmorstein et al., 2000; Qu and Hartzell, 2008; Rosenthal et al., 2006). One of the hallmarks of BEST disease is the accumulation of fluid-filled lipofuscin deposits in the sub-retinal space in between the RPE and the photoreceptors (Bakall et al., 2007; Mohler and Fine, 1981). It is thought that these deposits result from the accumulation of photoreceptor outer segments that have not been digested due to dysfunctional and diseased RPE. In a recent report Singh *et al* used BEST-patient-specific iPS cell-derived RPE to delineate some of the disease cellular endophenotypes in RPE cells (Singh et al., 2013b). Authors showed *in vitro* that, consistent with the patient phenotype, these RPE cells are slow in digesting photoreceptor outer segments (POS) and have defective ability to transport fluid from apical to basal sides. Authors link these cellular endophenotypes to potential abnormalities in calcium homeostasis in patient-derived cells. However, they do not provide a direct link between the mutant BEST1 protein, calcium homeostasis, and cellular endophenotypes. Clearly, more work is needed to further understand the molecular basis for pathophysiology of *BEST1* mutations. This work, nevertheless, provides an important milestone for the use of patient-specific iPS cells for *in vitro* disease modeling of retinal degenerative diseases.

In another example, Yang *et al* demonstrated that it is indeed feasible to model certain aspects of polygenic and complex diseases like AMD using patient-specific iPS cell-derived RPE (Yang et al., 2014). AMD is a polygenic disease and a number of risk alleles/loci have been associated with it (Fritsche et al., 2014). However, one big gap in our understanding of AMD pathophysiology is the inability to biologically associate these risk alleles with

specific molecular pathways. Yang *et al.* were able to validate the pathological nature of a set of these risk alleles in RPE derived from patient-specific iPS cells. They generated RPE from an AMD patient's iPS cells that were genotyped for three genetically-linked AMD haplotypes at ARMS2/HTRA1 locus (rs10490924, InsDel, and rs11200638) (Kanda et al., 2007). In an unbiased proteomic screen using a well-characterized RPE stressor A2E, (see Sparrow et al., 2003 for more details), authors identified reduced SOD2 activity and defective oxidative stress response in these high-risk allele containing iPSC-RPE cells as compared to cells that contain the low-risk haplotype. The study, however, did not attempt to connect these risk alleles molecularly to the defective oxidative stress response that was seen in patient's cells. Furthermore, these findings need to be reproduced on additional patient samples with identical ARMS2/HTRA1 risk-loci. Despite some of these drawbacks, this work clearly provides a mechanism to understand the role of AMD risk alleles and identify pathways that can be therapeutically targeted. Because AMD is associated with multiple different risk alleles and is likely associated with multiple different signaling pathways (Fritsche et al., 2014), a population genetics approach will be needed in future to identify the contribution of additional pathways and risk alleles to AMD pathology.

In addition to the two studies discussed here (Singh et al., 2013b; Yang et al., 2014), a number of other studies have discussed other forms of retinal degenerative diseases (Cereso et al., 2014; Li et al., 2014; Polinati et al., 2014). Combined together, all these studies have provided the critical first steps that were needed to demonstrate the utility of patient-specific iPS cells for *in vitro* disease modeling. However, several key details are still lacking from these disease models. Both BEST disease and AMD affect the macula (the central part of the eye) and it is well accepted that macular-region and peripheral-regions of the eye contain distinct types of RPE cells that differ in structure, gene expression, and cellular function (Bowes Rickman et al., 2006; Cai et al., 2012; Newman et al., 2012; Sharon et al., 2002; van Soest et al., 2007). Macular RPE cells predominantly interact with bright-light-sensitive cone photoreceptor cells, whereas peripheral RPE cells interact with dim-light-sensitive rod photoreceptor cells. Furthermore, both these diseases affect the entire homeostatic unit in the back of the eye, whereas these initial studies focused mainly on the cell autonomous phenotype in RPE cells. It is thought that three-dimensional cultures of RPE, retina, and choroid will provide more insight in to disease pathophysiology and allow the possibility of developing macular v/s peripheral disease models.

Three-dimensional Models of Retinal Degenerative Diseases

Ongoing efforts utilize developmental biology and tissue engineering approaches to build three-dimensional RPE-photoreceptor tissues. In this approach initiated by the seminal work performed by Eiraku *et al.* in 2011, RPE and photoreceptor cells are co-developed from mouse ES cells. These authors demonstrated the self-organizing capacity of mouse ES cells to form a three dimensional optic cup that developed into a retina containing all the major retinal neurons that differentiate in a temporal pattern reminiscent of the normal mammalian retina development (Eiraku et al., 2011). This work was soon reproduced by the same group using human iPS cells and independently produced by the Gamm lab, followed by others (Nakano et al., 2012; Phillips et al., 2012; Reichman et al., 2014). Subsequent studies improved upon the initial protocols and demonstrated an outgrowth of outer segments in the

photoreceptor layer in long-term cultures (Zhong et al., 2014). This is considered an important break-through, because several previous reports that generated photoreceptors from human or mouse pluripotent stem cells could not demonstrate the formation of photoreceptor outer segments, the main light-sensing part of these cells. Although in all these cases optic cup growth begins with an MITF and PAX6 co-expressing RPE-like tissue that lines the 3D retina, the RPE layer does not develop into a mature and functional tissue. In fact, often the RPE layer is not properly maintained in culture due to technical limitations of differential culture methods used for RPE and neuroretinal cells. Therefore, currently, this approach mainly provides a potential tool to develop disease models relevant for the retina and significantly more work is needed to develop co-cultures of RPE, photoreceptors, and the choriocapillaris.

The developmental approach discussed above can be complemented by a tissue engineering approach. In this approach, the goal of RPE and photoreceptor co-culture is achieved by first growing them separately and then layering the photoreceptor layer on top of the RPE using artificial scaffolds. McUsic *et al.* attempted this approach with small success by guiding growth of dissociated mouse native retinal cells on top of an RPE monolayer (McUsic et al., 2012). Rod photoreceptors extended what looks like rhodopsin-expressing outer segment disc-like domains. Again, long-term co-cultures could not be established, but this approach has the potential for success if longer-term co-cultures of the two cell types can be established.

Another possible way the problem of co-culture has been solved uses organ-on-a-chip technology (Esch et al., 2015; Huh et al., 2011; Sung et al., 2014). This microfluidic technology allows recapitulating some of the key functional aspects and interactions of a tissue in a microfluidic platform. One of the most well-known microfluidic device is called the “lung-on-a-chip” that allows culture of alveolar epithelium and endothelium cells on the two sides of a semi-permeable membrane, allowing different culture medium to be used for each cell type. Lung-on-a-chip has successfully recapitulated barrier function between alveolar epithelial cells and endothelial cells (Huh et al., 2010). Microfluidic devices allow precisely controlled flow rate in physiological conditions, which mimics *in vivo* organ functioning. It is well known that flow-induced shear stress affects vascular permeability and other functions of endothelial cells (Creighton, 2013; Orsenigo et al., 2012; Zhou et al., 2014). Similarly, a “heart-on-a-chip” has been also introduced to generate contractions in myocytes seeded on a thin film by electrical stimulation. This device has been used for high-throughput drug screening (Grosberg et al., 2011; Lee et al., 2014), and disease modeling. Patient-specific iPS cell-derived myocytes have been grown in microfluidic platform to develop an *in vitro* model of Barth syndrome, a congenital hypertrophy of heart caused by mutations in an acyltransferase gene. So far, no such models exist for ocular cell types. As discussed above, both RPE and photoreceptors have been developed from iPS cells, but no attempts have been made to obtain choroid-specific endothelial cells. But, it is not even clear if choroid-specific endothelial cells are actually required for this tissue chip approach. Multiple reports have developed generic endothelial cells from human ES and iPS cells (Orlova et al., 2014; Patasch et al., 2015). It is possible that a co-culture of RPE with generic endothelial cells will induce a choroidal-endothelial phenotype in these cells. Thus, it is hoped that a combination of developmental biology and tissue engineering approaches will

provide a three-dimensional co-culture of RPE, endothelial cells, and photoreceptors where complete photoreceptor outer segments can be developed. This tissue will likely serve as a more effective *in vitro* retinal degenerative disease model compared to RPE cells or retinal cells alone.

Current and Future Strategies for RPE Cell Transplantation

Current efforts to develop a cell replacement therapy for AMD using pluripotent stem cell-derived RPE is supported by a long history of autologous RPE transplants in AMD patients. Successful transplants have been done using autologous RPE/choroid grafts cut out from the peripheral portion of same patient's eye (Joussen et al., 2007; Maaijwee et al., 2007; van Zeeburg et al., 2011). This concept has provided hope that autologous RPE patch derived from patient-specific iPS cells can provide visual benefits for the patients. Before the discovery of iPS cells, the initial effort to develop replacement RPE tissue focused on allografts developed from human ES cells. Two main strategies are being used for transplanting RPE cells in the sub-retinal space (Table 2). In the first case, RPE cells are injected as a “bolus” in cell suspension designated as the “transient dosing” strategy. In the second case, RPE cells are transplanted as a polarized monolayer cell sheet, with or without an artificial scaffold support, designated the “permanent implantation” strategy. Table 2 summarizes some of these ongoing efforts that are using these two strategies. As the name suggests, the “transient dosing” strategy is not thought to provide a long-term engraftment of cells. There is limited evidence that an injection of RPE cell suspension can form a confluent and polarized RPE monolayer in the subretinal space and integrate into the host's Bruch's membrane (Diniz et al., 2013). Most pre-clinical animal data suggests that cells often clump and evoke an innate immune response that leads to their cell death (Carr et al., 2009b; Diniz et al., 2013). Furthermore, a cell suspension cannot perform several RPE functions that a polarized cell layer can perform. For instance, a cell suspension can't secrete cytokines in a polarized fashion; can't transport fluid vectorially from apical to basal surface of RPE monolayer; and can't carry out specific receptor-mediated phagocytosis of photoreceptor outer segments. Because of some of these deficiencies the “transient dosing” strategy mainly relies on the ability of an RPE cell suspension to secrete neuroprotective factors in a non-polarized fashion to protect the photoreceptor cells from dying. Despite the fact that the “transient dosing” strategy is scientifically not very compelling, one major advantage is the relatively low cost of cell manufacturing and transplantation as compared to some of the autologous iPS cell-based strategies.

Recently, a stem cell-based company Advanced Cell Technology has reported successful completion of two phase I/IIa clinical trials for ES cell-derived RPE cell suspension transplanted in AMD and Stargardt's disease (Schwartz et al., 2012; Schwartz et al., 2015). In both these trials authors performed a three-cohort dose escalation study with 50000, 100000, and 150000 cells in each cohort. In the highest dose, authors reported a few cases of micro peri-retinal cell growth, which likely happened due to cell leakage from the sub-retinal space into the vitreous cavity. But overall, both the trials were considered to have met safety endpoints. Over a median follow up of 22 months, there were no major adverse events or immune-reactions noted in any of these 18 patients. This is a first trial of its kind and it provides hope that stem cell-based therapies for retinal degenerative diseases are possible.

In contrast, to the “transient dosing” strategy the “permanent implantation” strategy intends to transplant RPE monolayer that may engraft in the host Bruch's membrane, perform all the functions performed by native RPE cells, and provide a longer-term implant. In this strategy, RPE cells are grown as a polarized monolayer on an artificial scaffold *in vitro* before transplantation (Lu et al., 2014; Stanzel et al., 2014). Multiple different substrates have been tried for generating the RPE tissue (Jha and Bharti, 2015). For instance, RPE monolayer tissue grown on polyester or paralene substrates are being developed for clinical use (Lu et al., 2014; Ramsden et al., 2013). Both these materials are non-degradable, but allow a hard implant that has been successfully transplanted in preclinical animal models (Hu et al., 2012; Stanzel et al., 2014). As the next generation of scaffolds, several labs including our group are testing biodegradable poly-lactic-co-glycolic acid (PLGA) materials with the goal of allowing the transplanted RPE tissue to completely integrate into the host Bruch's membrane (Liu et al., 2014). We have optimized the fiber diameter and pore size to obtain a RPE monolayer on top and have also optimized fiber degradation to obtain sufficient physical strength in the scaffold needed for transplantation purposes (Fig. 2). Furthermore, we have functionally authenticated the RPE monolayer that forms on top of the PLGA scaffold and shown that iPS cell-derived RPE acquire several key functional properties of native RPE cells. For example, Figure 2 shows a TEM of iPS cell-derived RPE on a PLGA scaffold. Cells retain key morphological RPE features like extensive apical processes, basal infoldings, apically localized melanosomes, and tight junctions between adjoining cells. Figure 3 shows that RPE monolayer on PLGA scaffold has the ability to transport water from apical (subretinal) to basal (choroidal) side. The RPE monolayer is electrically intact and has transepithelial resistance of $>150 \Omega \cdot \text{cm}^2$ and transepithelial potential of 2.0–2.6 mV (Maminishkis et al., 2006; Rizzolo, 2014). Currently, we are testing the safety and efficacy of these RPE scaffolds in animal models.

To date, the only patient that has been transplanted with a stem cell derived RPE monolayer tissue used an autologous iPS cell-derived RPE tissue that was manufactured without an artificial scaffold (Kamao et al., 2014; Reardon and Cyranoski, 2014). RPE cells grown on a collagen substrate were enzymatically lifted and transplanted in a patient with advanced CNV (Reardon and Cyranoski, 2014). This work shows that the approach of monolayer tissue transplantation is feasible. One advantage of the “permanent implantation” strategy as compared to the “transient dosing” strategy is that cells will likely not migrate away from the area of the transplant. But, dosing in the case of “permanent implantation” strategy is limited to the size of the scaffold used for transplantation and it may not be easily feasible to increase the dosage in this case. The two currently approved potential “permanent implantation” strategies, however, utilize scaffolds that cover almost the entire macula (see www.Clinicaltrials.gov for more details). Ongoing and future studies will show whether the use of artificial scaffolds (degradable or non-degradable) provide better survival and engraftment of RPE tissue as compared to RPE monolayer without any scaffold or the injection of RPE cell suspension.

Clinical Challenges in RPE Transplantation

Use of pluripotent stem derived RPE in clinical application has multiple challenges, including manufacturing of clinical-grade cells, potential tumorigenicity of transplanted cells, and immune-acceptance of transplanted cells.

Four different INDs utilizing ES cell derived RPE (Table 2) have been approved. This suggests that robust Good Manufacturing Practice (GMP)-compliant protocols for culturing of ES cells and their derivation to RPE currently exist. The same is not true for iPS cell derivation and differentiation into RPE. There have been a number of protocols developed for the generation of transgene, virus, proto-oncogene, and xeno-free iPS cells (Chou et al., 2015; Goh et al., 2013; Okita et al., 2008). Similarly, protocols have been published to generate RPE under xeno-free conditions from pluripotent stem cells (Pennington et al., 2015; Sridhar et al., 2013). However currently, there are no published or academically-available protocols to derive iPS cells or to differentiate iPS cells into RPE under GMP-compliant conditions. Significant effort is being put forward in this field and it is expected that in the near future GMP-grade iPS cell lines and protocols to develop new iPS cell lines under GMP-compliant conditions will be made available. At this early stage of iPS cell-based clinical efforts, it is critical to overcome some of these logistic hurdles to generate GMP-compatible protocols both for generating iPS cells and their differentiation into RPE.

The pluripotent nature of ES and iPS cells also raises the concern that if any non-differentiated pluripotent stem cells were left in the final clinical product, they could increase the risk of tumor or teratoma formation after transplantation. This possibility is further underscored by the recent observation of potential tumorigenic mutations in some of the clinical-grade iPS cell lines derived from one AMD patient as part of a clinical study at the RIKEN institute in Japan (Garber 2015). Although the recently completed phase I/IIa trials of Advanced Cell Technology (Schwartz et al., 2012; Schwartz et al., 2015) diminishes some of these concerns, especially for ES cells, utmost care must be taken in selecting ES and iPS cells that do not carry any potential tumorigenic mutations.

As some of these initial clinical efforts become successful in the near future, three strategies can help expand the scope of iPS cell-based therapies to a larger population and to multiple diseases: (1) *Ex vivo* gene therapy involves correcting disease causing mutations in patient-derived iPS cells before using those cells for a cell therapy application. Multiple techniques have been developed recently for gene editing including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas system (Pattanayak et al., 2014). In monogenic diseases, especially diseases that are genetically dominant, *ex vivo* gene correction will be required to generate an effective cell therapy (for more details on *ex vivo* gene therapy see Burnnigh et al., 2015). (2) Human Leukocyte Antigens (HLA) are the main determinant of an allogeneic immune response (Thomas et al., 2015). Most individuals are heterozygous for the three major HLA-A, HLA-B, and HLA-DR loci that are randomly distributed across a given ethnic population (Goldberg and Rizzo 2015). It is thought, iPS cells derived from individuals that are homozygous for a given allele can be used to develop cell therapy product that might be immune-compatible with a relatively larger fraction of the population. For instance, it is expected that 100 iPS cell lines that are homozygous for

different HLA-haplotypes will provide a match for at least one HLA-A, HLA-B, or HLA-DR loci for up to 80% of European-American population (Gourraud et al., 2012; Taylor et al., 2012; Zimmermann et al., 2012). Therefore, attention needs to be given to GMP-grade HLA-matched haplo-banks of iPS cells. In fact, global efforts are ongoing to compile a database of such HLA-homozygous donors among different populations (Turner et al., 2013). At this stage, it is, however, not clear if HLA-matching at these three loci will provide complete immune privilege for transplanted iPS cell derivatives. It is possible that in certain tissue other rare HLA-alleles can evoke an allo-immune response (Gourraud et al., 2012). (3) A slightly different and perhaps logistically and financially less burdensome strategy would be to manufacture a universal donor iPS/ES cell line that is homozygously deleted for all three of the HLA-alleles (Riolobos et al., 2013). Lab-grade efforts suggest that such embryonic stem cells can be derived by genetic engineering and their derivatives can evade immune response when transplanted into a host (Riolobos et al., 2013). But, such a line poses additional regulatory hurdles of combining *ex vivo* genetic engineering with pluripotent stem cell technology. Hopefully, in the near future FDA might allow approval of this technology for clinical applications.

Conclusions

iPS cells have been successfully and reproducibly differentiated into functional RPE cells. Patient iPS cell-derived RPE cells have been used for proof-of-principle “disease modeling” where disease associated cellular endophenotypes have been reproduced in the cells. However, in many cases a direct association between the disease causing genetic mutation, the cellular endophenotype, and the clinical phenotype is lacking. The ability to generate patient-specific iPS cells provides a direct access to patient's genetics and allows the possibility of discovering these causative links between genotype, cellular endophenotype, and the clinical phenotype. This also allows the possibility of discovering disease-initiating molecular pathways that can provide more effective targets for therapeutic intervention.

Tissue engineering combined with iPS cell technology and developmental biology will provide the basis for developing three-dimensional models of the RPE, neuroretina, and the choriocapillaris. Analysis of disease processes at the level of this entire homeostatic unit will likely provide more insight into molecular mechanisms of retinal degenerative diseases. Multiple approaches that utilize either no scaffold or an artificial scaffold to form an RPE monolayer from iPS cells are being tested as potential cell replacement therapies for retinal degenerative diseases. Currently, iPS cell-based therapies are being tested as autologous tissue. In future, development of HLA-homozygous iPS cells with GMP protocols will allow the possibility of testing RPE transplantation across a broader population.

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Highlights

- Current progress in differentiation of iPS cells into functional RPE cells
- Two and three-dimensional *in vitro* models of retinal degenerative diseases
- Transplantation of iPS cell derived RPE

Figure 1. Schematic diagram of the ocular homeostatic unit in ocular tissue (not to scale): (A) Healthy eye (B) Intermediate “dry” AMD stage targeted for in vitro system (C) Advanced “dry” AMD stage targeted for cell therapy (D) “Wet” form age related macular degeneration targeted for 3D disease modeling.

Figure 2.

Transmission electron micrograph of iPS cell derived RPE cells growing on a poly-(lactoco-glycolic acid) (PLGA) scaffold. RPE cells contain several of the known morphological features of native RPE like apically located pigment granules (melanosomes), extensive apical processes, tight junctions between neighboring cells, and infoldings in the basal membrane. Scaffold fibers are also visible underneath RPE cells.

Figure 3.

iPS cell-derived RPE monolayer grown on a PLGA scaffold transports water from its apical to basal sides. RPE monolayer exhibits electrical properties similar to the native human RPE with transepithelial resistance (RT) between $180\text{--}200\ \Omega\cdot\text{cm}^2$ and transepithelial potential (TEP) around 2 mV. The basal fluid absorption rate (apical to basal) of RPE monolayer is less than $1\ \mu\text{l}\cdot\text{cm}^{-2}\cdot\text{hr}^{-1}$. This fluid flow increases to $3\text{--}4\ \mu\text{l}\cdot\text{cm}^{-2}\cdot\text{hr}^{-1}$ because of ATP addition to the apical bath. ATP activates purinergic receptors on RPE apical surface, which mobilizes intracellular calcium stores to activate calcium-activated chloride channels on the basal side and iso-osmotically drive fluid from apical to basal RPE side.

Table 1

Different approaches used to differentiate 2D RPE and retinal cells, and 3D ocular tissue from human pluripotent (ES and iPS)

stem cells					
	Stem cell type (source)	Ocular cell type	Duration	Differentiation strategy	References
1. Spontaneous differentiation of pluripotent stem cells to RPE					
I	ESCs (monkey)	RPE cells	3–4 months	Pigmented RPE clusters are manually or enzymatically purified to obtain relatively pure RPE cells	Kawasaki et al 2002; Haruta et al 2004; Klimanskaya et al 2004; Lund et al 2006; Vugler et al 2008, Carr et al 2009a; Buchholz et al., 2009; Carr et al 2009b
II	ESCs (human)	RPE cells	3–4 months		
III	iPSCs (human)	RPE cells	3–4 months		
2. Step-wise differentiation of pluripotent stem cells to retinal progenitors and photoreceptors					
I	I ESCs (human)	Retinal neurons	3 months	WNT and TGF antagonist promote retinal progenitor differentiation.	Lamba et al 2006
II	ESCs/iPSCs (mouse/human/swine)	Photoreceptor, RPE cells	1–5 month	Photoreceptor differentiation promoted by RA, Shh, and Taurine	Osakada et al 2008, 2009a, 2009b; Reh et al 2010; Hiram et al., 2009; Meyer et al 2009; Zhou et al 2011
3. Step-wise differentiation of pluripotent stem cells to retinal progenitors and RPE					
I	I ESCs (human)	RPE cells	8–10 weeks	ACTIVIN A	Idelson et al. 2009
II	ESCs/iPSCs (human)	RPE cells	2–8 weeks	ACTIVIN A, SU5402, VIP	Buchholz et al 2013
III	iPSCs (human)	RPE cells	8–10 weeks	Dual-SMAD inhibition and ACTIVIN A	Ferrer M. et al. 2014
IV	ESCs (human)	RPE cells	2–8 weeks	WNT agonists	Leach et al 2015
4. 3D differentiation of pluripotent stem cells to ocular tissue					
I	I ESCs (mouse)	3D retina/RPE	2–5 weeks	Autonomous patterning	Eiraku et al 2011
II	II ESCs (human)	3D retina/RPE	3–18 weeks	Autonomous patterning; WNT for RPE; NOTCH, RA, Taurine for photoreceptors	Nakano et al 2012; Gonzalez-Cordero et al 2013
III	iPSCs (human)	3D retina/RPE	3–21 weeks	Autonomous patterning, proneural medium for retina	Philips et al 2012; Reichman et al 2014; Zhong et al. 2014

Abbreviations: ESCs – Embryonic Stem Cells; iPSCs – Induced Pluripotent Stem Cells; RPE – Retinal Pigment Epithelium; RA – Retinoic Acid

Table 2

The two different transplantation strategies used for ES and iPS cell derived RPE for AMD patients

	Investigator/company	Starting Cells	Immune status	Transplantation vehicle	Clinical stage
1. Transient dosing strategy					
I	Advanced Cell Technology (ACT)	ESCs	Allogeneic	Cell suspension	Phase I/IIa completed
II	Cell-cure-neurosciences (Eyal Banin & Benjamin Reubinoff, HMC)	ESCs	Allogeneic	Cell suspension	IND approved, Phase I initiated
2. Permanent implantation strategy					
I	Pfizer, London (Pete Coffey, UCL)	ESCs	Allogeneic	Polyester scaffold	IND approved, Phase I initiated
II	PI - Mark Humayun (USC), Co-PIs - Dennis Clegg (UCSB) & David Hinton (USC) (CIRM, USA)	ESCs	Allogeneic	Paralene scaffold	IND approved, Phase I initiated
III	Masayo Takahashi (RIKEN, Japan)	Fibroblasts-derived iPSCs	Autologous	RPE sheet, no scaffold	First patient transplanted, study halted
IV	Kapil Bharti & Sheldon Miller (NEI, NIH, USA)	CD34+ cell-derived iPSCs	Autologous	Biodegradable scaffold	Performing IND-enabling studies

Abbreviations: AMD – Age-related Macular Degeneration; ESC – Embryonic Stem Cells; IND – Investigational New Drug; iPSC – Induced Pluripotent Stem Cells; RPE – Retinal Pigment Epithelium

Clinical Groups Affiliations: UCL – University College London; USC – University of Southern California; UCSB – University of California Santa Barbara; HMC – Hadassah Medical Center; NEI – National Eye Institute; NIH – National Institutes of Health