Application of stem cell-derived retinal pigmented epithelium in retinal degenerative diseases: present and future

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Abstract

• As a constituent of blood-retinal barrier and retinal outer segment (ROS) scavenger, retinal pigmented epithelium (RPE) is fundamental to normal function of retina. Malfunctioning of RPE contributes to the onset and advance of retinal degenerative diseases. Up to date, RPE replacement therapy is the only possible method to completely reverse retinal degeneration. Transplantation of human RPE stem cell-derived RPE (hRPESC-RPE) has shown some good results in animal models. With promising results in terms of safety and visual improvement, human embryonic stem cell-derived RPE (hESC-RPE) can be expected in clinical settings in the near future. Despite twists and turns, induced pluripotent stem cell-derived RPE (iPSC-RPE) is now being intensely investigated to overcome genetic and epigenetic instability. By far, only one patient has received iPSC-RPE transplant, which is a hallmark of iPSC technology development. During follow-up, no major complications such as immunogenicity or tumorigenesis have been observed. Future trials should keep focusing on the safety of stem cell-derived RPE (SC-RPE) especially in long period, and better understanding of the nature of stem cell and the molecular events in the process to generate SC-RPE is necessary to the prosperity of SC-RPE clinical application.

• KEYWORDS: retinal pigmented epithelium transplantation; stem cell; human embryonic stem cell; induced pluripotent stem cell

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INTRODUCTION

The integrity of retinal pigmented epithelium (RPE) is vital to normal retinal function. The tight junction between RPE cells is a main constituent of blood-retinal barrier, which allows nutrients to diffuse into retina, and metabolites to be transported outward to the choroid; besides, RPE cells participate the retinol cycle and clear retinal outer segments (ROS) through phagocytosis[1-2]. RPE degeneration interferes with normal retina metabolism, breaks the blood-retina barrier, and finally causes vision loss.

Retinal degenerative diseases affect patients of all age groups. Age-related macular degeneration (AMD) is a very common disease among elderly people. About 80%-90% of them are atrophic or dry AMD characterized by drusen, RPE and photoreceptor degeneration and geographic atrophy[3-4]; wet AMD, or exudative AMD, is associated with abnormal neovascularization, leading to exudation, hemorrhage and subsequent fibrosis. While intravitreal injection of anti-vascular endothelial growth factor (VEGF) agents can effectively mitigate neovascularization of exudative AMD in many cases, some don’t react well, and there is no effective intervention for atrophic AMD, especially in advanced stage[2]. Some studies indicated that alterations of RPE and choroid were prior to retina. With the common knowledge that abnormal RPE accelerate photoreceptor degeneration[5], it is reasonable to postulate that the retinal lesion is a result of RPE pathological changes. Stargardt disease (STGD), unlike AMD, is an autosomal recessive macular dystrophy whose onset is common in childhood and early adulthood[6]. In most cases, gene defects of ABCA4 result in accumulation of bisretinoid A2PE in ROS, which hydrolyzed to highly toxic A2E, and thus lead to RPE dysfunction. In return, RPE failure contributes to subsequent photoreceptor dysfunction/death. Bestrophinopathies are a collection of diseases with various BEST1 mutations, affecting mostly retina and in some cases, vitreous and choroid[7]. Accumulation of lipofuscin in RPE is a mutual pathological change shared by Best vitelliform macular dystrophy (BVMD) and autosomal recessive bestrophinopathy (ARB), the most common and apprehended macular dystrophies of bestrophinopathies. In these retinal degenerative diseases, initial or subsequent RPE lesions inevitably contribute to photoreceptor deaths.
To date, no effective treatment can completely reverse RPE degeneration. Since RPE is not self-renewable, it’s logical to replace diseased RPE with functioning substitute. Different strategies for autologous RPE transplantation were explored. It has long been proven since 1990s that transplantation of human RPE (hRPE) to animal models early in postnatal life preserves photoreceptors from death\[8\]. ERG shows rescued a- and b-waves\[9\]. Even at a later stage when RPE degeneration has advanced, hRPE also exhibits some protective effects after transplantation\[10\]. Phillips et al\[11\] first attempted autologous transplantation of health peripheral RPE to diseased area in rabbits, and observed protective effects to choroid and photoreceptors at transplanted sites. Besides, numerous studies have confirmed a trophic effect of transplanted RPE mediated by pigmented epithelium-derived factor (PEDF) and other factors since the rescue effect was observed beyond transplant limits\[12\]. Later studies extended the recognized protection effect of photoreceptors to synaptic connectivity and light transduction circuitry in animal models with pre- and post-synaptic markers immunostaining\[9\]. These results clarify the rationality of RPE replacement therapy. However, limited source, high risk of surgical complications and internal genetic defects of most diseases hinders application of autologous peripheral RPE transplantation\[2\].

Stem cell therapy avoids major drawbacks of autologous RPE transplantation by nature. Stem cells can be categorized in different manners. By differential potency, they can be classified into totipotent, pluripotent and multipotent stem cells\[13\]. Totipotent stem cells are able to differentiate into both embryonic and extra-embryonic tissues. Pluripotent stem cells (PSC) can differentiate into embryonic tissues. Multipotent stem cells are only able to form specific tissues. By origin, stem cells can be harvested from inner cell mass, fetal tissues or adult organs such as liver and brain, thus named embryonic, fetal and adult stem cells respectively. Embryonic stem cells (ESC) are usually pluripotent. Fetal stem cells are less potent but still play a role in some clinical scenarios. Adult stem cells disperse in major organs such as skin, intestinal epithelia and brain, which is the basis of tissue self-repair\[13\]. As for the retina, recent researches have identified a minor subgroup of hRPE cells that can be stimulated to a stem cell state in which the cells lose RPE markers, and regenerate RPE and mesenchymal progenies in vitro\[14\]. However, their application is impeded by limited potency and unproportionate difficulty of access. In 2006, Takahashi and Yamanaka\[15\] transfected fibroblasts with retrovirus vectors to express four transcriptional factors (TF), Oct3/4, Klf4, Sox2 and c-Myc (OKSM), yielding ESC-like cells, namely induced pluripotent stem cells (iPSC). This technique opened a new era of stem cell therapy and made Takahashi and Yamanaka later Nobel laureates.

Regeneration of diseased tissue with both structural and functional restoration is a core concept of stem cell therapy. ESC and iPSC are two most employed stem cells to generate RPE (hence ESC-RPE and iPSC-RPE respectively). Although both of them are pluripotent and proven able to generate RPE, their unique features make each type irreplaceable. Firstly, unlike ESC, it is necessary that somatic cells go through reprogramming process in order to regain pluripotency and become iPSC. Initially, the essence of most methods was to deliver exogenous TF into cells, either through viral transfection or chemical-based delivery such as nanocarriers\[16\]. Widely-used viral vectors include integrating and non-integrating ones. The former, despite being the earliest to generate iPSC\[15\], doesn’t meet clinical demands due to inevitable risk of mutagenesis associated with viral genome integration. The latter, although genetically stable, can be immunogenic to various degree, even life-threatening in some cases\[16\]. Besides, both vectors have a tendency to be rejected by host cells, either by uncontrolled gene silencing or direct ejection of vectors\[17\]. Unexpectedly, exploration of small molecules (SM) in their role of improving viral-induced reprogramming efficiency unveiled their potential to reprogram somatic cells, even free of transgenes\[18\]. Current interest has shifted from TF gene delivery to step-wise chemical induction with defined SM, which is easily standardized, theoretically non-immunogenic and can be manipulated in terms of concentrations, treatment durations, combinations, etc, to optimize their effects\[18-19\]. This process is mediated by an extraembryonic endoderm (XEN)-like state with characteristic gene expression and morphology\[19\] instead of a primitive streak-like state reported in OKSM-induced reprogramming\[20\], indicating distinct molecular events between the two reprogramming methods. Various cell types have generated chemical induced pluripotent stem cells (CiPSC), such as fibroblasts from mesoderm, neural stem cells from ectoderm and intestinal epithelial cells from endoderm with accordingly modified SM cocktails\[21\], which were able to form teratomas containing tissues of all three germ layers and generate chimeric mice. Although to our knowledge, no such CiPSC have been reported to generate RPE. Their promising future in clinical application attracts intense interest and investigation.

Secondly, although subretinal space is considered an immune-privileged site, the immune environment especially under disease condition with blood-retinal barrier defects remains poorly understood. Natural RPE can suppress both innate and adaptive immune system by secreting various combination of cytokines, such as transforming growth factor (TGF)-β, PEDF, somatostatin, etc\[2\], which orchestra cell signaling pathways. Although immunological properties of stem cell-derived RPE (SC-RPE) can’t be simply postulated by analogy to its natural.
epigenetic memory

iPSC might inherit patients’ own genetic vulnerability or bear ocular sites, which is distinct from high immunogenicity of immunogenic antigens. Yet the result encourages iPSC-RPE allografts survive and function well with immunosuppression. With proper management, ESC and among others highlight the necessity of proper and adequate histology showed mononuclear cell infiltration around the graft border in rabbit eyes[23]. In vivo, allogenic iPSC-RPE suspension could elicit an innate T cell-mediated immune response marked with vitreous IL-12 elevation and subretinal infiltration of macrophage and leukocyte in unimmunosuppressed Yucatan mini-pigs[24]. It was suggested that major histocompatibility complex class I (MHC-I) and β2-microglobulin (β2-MG) protein expressed by human induced pluripotent stem cell (hiPSC) could be stimulated by interferon-γ (IFN-γ), a proinflammatory cytokine surgery may induce[25]. These results among others highlight the necessity of proper and adequate immunosuppression. With proper management, ESC and iPSC-RPE allografts survive and function well[26-27]. Theoretically, autologous iPSC-RPE transplantation escapes host immune rejection[28], thus avoids risks associated with immunosuppressive agents, such as infection and tumorigenesis at distant sites. However, conflicting results of syngeneic iPSC-derived tissue transplantation have made this notion disputable[29]. Zhao et al[30] found that iPSC generated by non-integrating viral-transfection were far less likely to form teratomas compared to their ESC counterparts when transplanted into syngeneic B6 mice, and that the scarcely-formed teratomas were infiltrated with CD4+ T cells together companied by tissue necrosis. Global gene expression analysis indicated this immunogenicity might derive from abnormally-high expression of two genes. The same group later demonstrated a tissue-specific immunogenicity[31] possibly related to various expression of immunogenic antigens. Yet the result encourages iPSC-RPE application in that iPSC-RPE are well-tolerated even at nonocular sites, which is distinct from high immunogenicity of iPSC-smooth muscle cells. Another major difference is that iPSC might inherit patients’ own genetic vulnerability or bear epigenetic memory[32] of initial differentiation and long-term exposure to environmental insults. In a word, each iPSC line exhibits unique features and potential of differentiation, the secrets of which hide and remain to be unmasked.

Besides iPSC, retinal pigmented epithelium stem cell (RPESC) showed protective effect of photoreceptors[34] and vision[35] in a rodent model. Since hRPESC are closer to RPE state than pluripotent hESC and hiPSC, it’s natural to postulate that they are more similar to nature RPE with less immunogenicity and tumorigenesis. Up to now, researches of RPESC-RPE are relatively scarce. With the potential advantages over its counterparts, RPESC-RPE may be a better option in terms of safety than its pluripotent counterparts in the future. In this article, we mainly describe methods to generate RPE from PSC and inspiration from both preliminary animal models and clinical trials. Generation of Retinal Pigmented Epithelium from Pluripotent Stem Cells Differentiation Plentiful studies have generated RPE from ESC or iPSC with different protocols[36-39]. Spontaneous differentiation into numerous types of cells initiates upon removal of fibroblast growth factor (FGF) from culture conditions[40], which takes weeks to months. Co-culturing stem cell on mitotically-inactive mouse embryonic fibroblasts (MEF) accelerates differentiation. Another three-dimensional (3D) embryoid body (EB)-mediated culture method is also widely adopted to avoid using MEFS and other types of feeder cells. Free-floating EBs are then planted on to extracellular matrix (ECM)-coated plate to encourage neuroectoderm fate[40]. Selection of pigmented cells is required for high yield of RPE in both culture methods, since initial differentiation is a non-selective process. An alternative directed differentiation method by artificial intervention of signaling pathways is combined with EB-mediated culture method to improve efficiency. This two-stage process first introduces neuralizing growth factors such as nicotinamide, Dkk-1 and Lefty-A to assist neuralization, and then furthers cell fate to RPE with other sets of growth factors[41-44]. Buchholz et al[45] reported a directed differentiation protocol that could convert nearly 80% of cultured hESC to RPE in 14d. With more thorough understanding of the complicated and vital role of Wnt signaling pathway, modification to the protocol with Wnt activation improved the conversion rate to around 97.7% at day 14 without manual selection or enrichment[3]. At present, directed differentiation is more favored by investigators, not only for higher efficiency, but also a better prospect of low-cost, standardized production. Leach et al[46] compared iPSC-RPE derived from 5 iPSC cell lines using spontaneous and directed differentiation methods. Spontaneous differentiation method with adherent culture failed to generate iPSC-RPE from 2 cell lines. Aside from that, no significant differences of iPSC-RPE were detected between the two differentiation methods in transcript levels, protein localization or functional analysis. Instead, variations of RPE65 and BEST1 transcription levels between cell lines indicated an internal lineage-specific difference.
Some studies obtain putative RPE from direct lineage conversion by transfecting lineage-determining transcription factors into somatic cells[47]. This method omits a pluripotent state thus is easier to manipulate, but again, safety and stability of RPE generated by viral transfection in this method remains to be further investigated.

**Retinal pigmented epithelium selection and enrichment**

As mentioned before, selection is necessary to obtain a pure culture of RPE. This is accomplished by manually picking up pigmented hexagonal cells or a two-step enzymatic isolation[7]. Manual selection employs 31-gauge needle or 25-gauge ophthalmic surgical knife to pick RPE from culture, and then trypsin is added to dissociate RPE cell cluster into single cells[52]. Enzymatic isolation consists two trypsin digestions, of which the first shorter one clears other loosely-attached cells, and the second longer one dissociates tightly-attached RPE[57]. Manual selection obtains RPE with higher purity, but more labor cost as well.

After selection, RPE cells are transferred to proper culture condition for enrichment. Matrigel, derived from extracellular matrix protein of murine sarcoma[41], provides a 3D environment where initial culture of stem cells or differentiated RPE can form abundant cyst-like structures[44]. To favor RPE formation, this 3D-culturing method can be combined with neuralizing factors and subsequent 2D culture to accelerate RPE monolayer formation[49]. Using this method, generated RPE were able to rescue photoreceptors upon transplanted to animals. However, application of animal feeder cells or other products has always been controversial in the field of stem cell especially in clinical settings due to unpredictability of non-human ingredients or unknown pathogens[48]. For example, hESC co-cultured with murine feeder cells express immunogenic non-human sialic acid[49]. In order to prevent potential risks of animal products, a defined xeno-free culture is preferred in clinical context despite longer period and higher cost. MEF can be replaced by human foreskin fibroblasts (hFF). KnockOutTM Serum Replacement (KO-SR) can substitute fetal bovine serum (FBS). As for matrix protein supporting RPE differentiation and enrichment, both natural ingredient found in Bruch’s membrane such as collagen, laminin, fibronectin, and vitronectin[50], as well as mature xeno-free commercial products[51] are optional. Up to now, some studies have successfully generated functioning SC-RPE without using FBS, murine feeder cells or Matrigel[52-53].

**Transplant formats**

After enrichment, SC-RPE are enzymatically dissociated into suspension which could be directly injected into subretinal space or seeded onto a scaffold to generate monolayer. Although only a small incision is required for injection of cell suspension, scattered RPE cells can’t reconstruct blood-retinal barrier unless integrating into host RPE and forming an intact monolayer. In some cases, cells may form isolated cell clumps, or even escape into vitreous, causing severe complications such as proliferative vitreal retinopathy[54-56]. Whereas polarized RPE monolayered-sheets resemble natural RPE structurally and functionally. The extracellular matrix and adhesion molecules prevent RPE from apoptosis[25]. When transplanted as sheets, RPE secrete more PEDF[56] and show a higher rate of survival and integration to host RPE[57] than as suspension. RPE sheets can be constructed with both biological scaffold such as gelatin, and synthetic or semi-synthetic ones. However, the homogeneity of biological or semi-synthetic products cannot be guaranteed[58], which makes defined artificial scaffold more suitable for production and clinical application. While some synthetic scaffolds are gradually degraded by hosts, others remain stable in subretinal space. Parylene is one such undegradable material widely used in RPE sheet construction. It can support RPE cells as an intact polarized monolayer without disrupting host RPE structure[4,57,59]. CPCB-RPE1, a mature commercial product of monolayered RPE supported by ultrathin (0.3-0.4 μm) vitronectin-coated parylene C film is currently being tested in a clinical trial (NCT02590692). Other degradable synthetic materials, such as poly(lact8ic-co-glycolic acid) (PLGA) and poly(e-caprolactone) (PCL), also embrace a promising future and are currently under intense investigation[60-61].

Besides scaffold-based methods, some studies generated a pure polarized RPE monolayer[62], for which collagen served as a temporary scaffold, and was degraded later before transplantation.

**Retinal pigmented epithelium characterization and functional analysis**

Some features are necessary to authenticate SC-RPE and their ability to compensate for lost functions. Structural features include pigmentation, polarity, tight junctions, which can be verified with transmission electron microscopy[29]. Gene and protein expression profile can be analyzed by RT-PCR, immunochemistry staining and Western blot. RPE-specific genes include but not limit to retinoid cycle-related RPE65 and CRALBP, phagocytosis-related MERTK, chloride channel-encoded BEST1[25]. Others such as differentiation-related genes and marker genes for non-RPE cells are also tested in some studies to illustrate differentiation status and purity[63]. Functional analysis of SC-RPE includes direct measurement of secreted molecules such as PEDF, phagocytic assay using purified ROS or foreign bodies, electrophysiological tests and animal behavioral observation. Besides electroretinogram (ERG), some studies evaluated transplanted SC-RPE performance by recording luminance threshold responses of superior colliculus and its histology[60,64]. Carr et al[40] assessed the integrity of retinal circuitry by measuring light-induced c-Fos expression. Most ESC-RPE and iPSC-RPE can be characterized by these aforementioned methods and exhibit various degree of function[42,65-66]. Optical coherence
tomography (OCT) and fundus photography enables direct visualization of transplant condition. With deeper comprehension of RPE physiology and disease pathology, methods better predicting in-situ SC-RPE performance are springing up.

Introduction of Preliminary Animal Experiments and Clinical trials

Embryonic stem cell-derived retinal pigmented epithelium

RPE replacement is the first therapy to possibly cure retinal degenerative diseases. Since the first generation of RPE from hESC[^154], various cell lines and protocols were intensely investigated together with morphology and transcriptional analysis. Technological advances undoubtedly solved RPE source problem to a large extent. Royal College of Surgeons (RCS) rats have long been considered a classical animal model for AMD research, which bears a spontaneous mutation of Mertk that results in failure of ROS phagocytosis, eventually RPE degeneration and photoreceptor death[^154]. Injection of hESC-RPE suspension was first explored. A plethora of studies have proved its protective effects on photoreceptors and visual function of RCS rats. Lund et al[^64] demonstrated that visual performance of hESC-RPE transplanted rats was significantly better than untreated controls. Accordingly, postmortem histology showed long-term preservation of transplanted hESC-RPE cells in subretinal space and extensive photoreceptor rescue in treated group. Lu et al[^27] observed that pigmentation level positively correlated with RPE specific gene expression but not with final visual performance, and that increasing transplanted cell counts within a certain range (5000 to 50,000 cells) gave better visual performance. OCT showed integration of RPE cells to host RPE. Tumorigenicity study proved long-term survival throughout life span (up to 8mo) without formation of teratomas or other tumors in NIH III mice, a immunodeficiency model devoid of T-cells, NK cells and T-independent B lymphocytes[^27]. While these experiments all transplanted RPE by trans-scleral subretinal cell injection, other groups made efforts to optimize hESC-RPE sheet delivery methods. Hu et al[^68] invented a plane device that holds a monolayer of hESC-RPE supported by 4 μm parylene substrate, which was transplanted into rat subretinal space via a trans-scleral-choroidal route commonly adopted in surgeries on small-sized eyes due to limited space for manipulation. The cell-seeded substrates were properly placed confirmed by OCT and histological examination. Post-surgical analysis showed that aside from minimal cell loss along substrate margins, the hESC-RPE layer remained intact. Using this technique, Thomas et al[^55] evaluated vision improvement of RCS rats and found that in addition to photoreceptor preservation, which was also elicited by mere scaffold transplantation probably due to surgical-induced release of neurotrophic factors, a specific rod rescue effect proven by superior colliculus electrophysiology was detected with CPCB-RPE1 transplant group. Interestingly, this effect only exhibited at positions over superior colliculus corresponding to transplanted area of retina, giving credits to hESC-RPE function. For larger eyes such as human eyes, pars plana vitrectomy followed by retinotomy, transplant delivery into subretinal space and silicone tamponade are involved. Brant Fernandes et al[^89] used a transplant injector to deliver CPCB-RPE1 which was curled to prevent cell loss into murine subretinal space. Similar strategy adopted in porcine experiments aiming to test surgical feasibility on eyes comparable to human showed encouraging results[^59]. Work of these authors not only provided preliminary safety evidence, but also shed light on refinements of protocol followed in clinical trials, such as approximation of cell amount to be transplanted, delivery method etc.

Schwartz et al[^56-72] transplanted hESC-RPE as suspension to human recipients for the first time following a dose-escalation protocol. The clinical trial recruited 9 STGD and 9 AMD patients with an average 22-month follow-up. No major complications such as hyperproliferation, tumor formation or transplant rejection were observed, which provided initial evidence for the safety of hESC-RPE transplantation in human. Yet some complications related to postsurgical immunosuppression and surgical procedure were observed, such as cataract progression and endophthalmitis. Although mainly intended to illustrate safety issues, the trials did provide inspiring results about efficacy of hESC-RPE transplantation. The 12-month post-transplantation follow-up showed that for the visual acuity of seven AMD patients, three eyes increased at least 15 letters, one eye improved 13 letters and three remained stable. For the visual acuity of seven STGD patients, three eyes increased at least 15 letters, three remained stable and one decreased more than ten letters[^71]. Fundus photography and OCT showed pigmentation and RPE thickening at transplanted sites, indicating successful integration of transplanted cells to host RPE. Similar fundus photography and OCT results were reported from another parallel trial with no major complications, which further lessened safety concerns and encouraged trials with less advanced disease[^73], although no significant visual improvement was demonstrated in this trial. At present, many ongoing clinical trials keep exploring the safety and various methods of hESC-RPE transplantation. For example, one study chose commercialized CPCB-RPE1 as transplant (NCT02590692). With more detailed data provided by these trials, hESC-RPE transplantation can be expected in clinical settings for RPE degenerative diseases in the near future.

Pluripotent stem cells-derived retinal pigmented epithelium

The revolutionary iPSC technique was proved able to generate putative RPE cells, and numerous studies have proved its authenticity regarding morphology, functional analysis and
behavior tests upon transplantation to animal recipients\textsuperscript{[37,74]}. hiPSC-RPE cell suspension exhibits protective effects of photoreceptors upon transplantation to RCS rats\textsuperscript{[73]}. Carr \textit{et al}\textsuperscript{[41]} also reported a rescued light-induced c-Fos expression in the inner nuclear layer (INL) and ganglion cell layer (GCL), which symbolized the integrity of retinal circuitry. Combined with gene editing, iPSC-RPE cells derived from a retinitis pigmentosa (RP) donor restored visual function of \textit{Mfrpdrd6}\textsuperscript{[75]} mice, a RP animal model\textsuperscript{[75]}. Another study drew a similar conclusion using \textit{Rpe65}\textsuperscript{rd12}/\textit{Rpe65}\textsuperscript{rd12} mice\textsuperscript{[74]}. Long-term survival was observed through lifespan with no tumor formation, indicating both safety and stability of these cells. Kamao \textit{et al}\textsuperscript{[25]} generated polarized monolayered iPSC-RPE sheets free of foreign bodies in order to best evaluate their in situ function, since polarity is essential to a great many ion channels, receptors and transporters orderly distributed on apical or basal side of RPE\textsuperscript{[62]}. They generated pure iPSC-RPE sheets by seeding suspended unpolarized cells onto type I collagen gel until a confluent pigmented monolayer was developed, then added collagenase to degrade supporting substrates. The iPSC-RPE sheets met aforementioned criteria of RPE characterization without immune rejection or tumor formation upon autologous transplantation to a primate model without immunosuppression. These studies among others project the importance of careful evaluation of stem-cell based treatment in future trials. There are two ongoing iPSC-RPE-based clinical trials (NCT02162953, NCT02464956) investigating bostonophanopathy and dry AMD respectively, which may provide valuable evidence of iPSC-RPE safety in the future.

**Genetic and Epigenetic Instability** Various factors contribute to genetic and epigenetic instability of stem cells, such as prolonged culture, specific culture conditions, replicative stress, \textit{etc}. Besides, aberrations of iPSC may be inherited from their somatic progenitors or acquired during reprogramming. Since there seems to be a pattern of abnormality occurrence in prolonged culture, it is hypothesized that some of them facilitate culture adaptation, such as chromosome number and structural variation\textsuperscript{[79]}, which are harbored more in iPSC than their ESC counterparts. For example, the most common aberration trisomy 12 alters global gene expression profile, and subsequently cell properties such as apoptosis, cell cycle, \textit{etc}\textsuperscript{[80]}, posing cells to tumorigenesis. Lamm \textit{et al}\textsuperscript{[81]} suggested that replicative stress and check point deficiency may lead to defective chromosome condensation and segregation, which was assisted by dysregulation of cytoskeletal genes. Garitaonandia \textit{et al}\textsuperscript{[82]} analyzed effects of various culture conditions to genetic and epigenetic integrity of hESC and hiPSC with extensive experiments and found enzymatic passembling and feeder-free culture were related to a higher rate of aberration. These results call for a Good Manufacturing Practicing-grade (GMP) protocol of reprogramming, culture and differentiation. Refinements of protocol will definitely improve the quality and safety of stem cells, but more understanding of molecular basis under these aberrations will tackle the problem from the root.

**Future Directions** Advances of basic stem cell research have endowed great potential to stem cell-based therapies. Decades of efforts have been translated to actual attempts in reversing retinal blindness with RPE replacement. Despite great deal of past achievements in basic science, animal experiments and clinical trials, more challenges remain in front. The potential of immunogenicity of allogenic ESC- and iPSC-RPE makes it requisite to implement proper immunosuppression. With the immunogenicity of autologous iPSC hidden in black box, there is a pressing need for more thorough understanding of iPSC nature. Besides, few remaining undifferentiated cells in the graft and prolonged culture period may give rise to tumorigenesis. Although related risks can be reduced with careful evaluation, a better clarified mechanism will prevent
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these adverse events from the root. Future study should investigate into different cocktails of culture conditions and their effects to cell genetic and epigenetic integrity with whole-genome sequencing, whole-genome methylation profiling and expression analyses, as well as look into the molecular basis behind these aberrations. The robust short-term safety data provided by hESC-based phase 1 or 2 trials encourages further investigation into timing of RPE replacement therapy, which may better function during the window between initiation of RPE degeneration and irreversible photoreceptor death. Follow-up of past and current trials will provide more valuable long-term safety data. Despite similarities with hESC in many aspects, iPSC-based therapies are hindered by genetic and epigenetic instability. With the maturation of more targeted mutation repair and genomic editing tools, it is likely to overcome this barrier as well as combine iPSC technology with gene therapy to combat some hereditary diseases. hRPESC-RPE may be another option to avoid the potential risk of immunogenicity and tumorigenesis. Standardized production protocol, clarified transportation and storage conditions and more safety data from animal experiments are prerequisite for future clinical trials. This is a time where promises come with challenges, yet the future of actually curing retinal degenerative diseases with stem cell-based therapies is foreseeable.

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