REVIEW ARTICLE

Induced pluripotent stem cells for retinal degenerative diseases: a new perspective on the challenges

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Abstract

Retinal degenerative diseases, including age-related macular degeneration and retinitis pigmentosa, are the prodominant causes of human blindness in the world; however, these diseases are difficult to treat. Currently, knowledge on the mechanisms of these diseases is still very limited and no radical drugs are available. Induced pluripotent stem (iPS) cells are an innovative technology that turns somatic cells into embryonic stem (ES)-like cells with pluripotent potential via the exogenous expression of several key genes. It can be used as an unlimited source for cell differentiation or tissue engineering, either of which is a promising therapy for human degenerative diseases. Induced pluripotent cells are both an unlimited source for retinal regeneration and an expectant tool for pharmaprojects and developmental or disease modelling. In this review, we try to summarize the advancement of iPS-based technologies and the potential utility for retinal degenerative diseases. We also discuss the challenges of using this technology in the retinology field.

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Retinal degeneration

The retina is part of the central nervous system and contains neurons that convert light signals into neural signals, which are then transmitted to the brain. Any defects involved in phototransduction and transmission in the retina will lead to visual impairment. Because the retina is located near the body surface and other anterior media (vitreous humour, lens, aqueous humour and cornea) are transparent allowing light acquisition, it can be directly observed by a funduscopic examination, or mediately visualized by a fluorescein angiography or optical coherent tomography. Currently, retinal disease is a major cause of inevitable blindness worldwide. Although diagnostic techniques for retinal disease have tremendously improved, the therapeutic techniques are still insufficient and limited.

Among the retinal degenerative diseases, age-related macular degeneration (AMD) is the leading cause of permanent blindness in the elderly, especially in developed countries. This disease affects the central area of the retina, which is essential for central and colour vision. AMD displays a broad spectrum of clinical and pathological abnormalities and can be classified into two groups: 'dry' and 'wet' AMD. 'Dry' AMD is characterized by atrophy and degeneration of the outer neural retina, retinal pigment epithelium (RPE), Bruch's membrane and choriocapillaris; whereas 'wet' AMD is characterized by subretinal neovascularization and related exudative maculopathy. Nevertheless, AMD disease is etiologically very complex. Although both environmental agents and genetic risk factors for AMD have been studied extensively, the underlying mechanism of the disease remains elusive. So far, great efforts have been made to explore new therapies for AMD including anti-vascular endothelial growth factor (VEGF) medicines and photodynamic therapy. These treatments could prevent the progress of neovascularization, a symptom of AMD; however, they could not repair the sensory retina and RPE once they are damaged.

Inherited retinal degeneration is a major cause of visual impairment in the juvenile-to-young adult population. In par-

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ticular, retinitis pigmentosa (RP) is the leading cause of inherited blindness or visual impairment. RP is a group of inherited retinal disorders characterized by night blindness and visual field defects caused by the loss of rod photoreceptors. RP encompasses a number of different manifestations with many distinct genetic causes and diverse biological defects, however, these diseases lead to photoreceptor degeneration and display some overlapping phenotypes. In fact, an increasing number of causative genes have been identified so far (see database in RetNet or Retinal International). Many of these genes are involved in the phototransduction cascade, vitamin A metabolism, structural maintenance, cell signalling or interaction (Hartong *et al*. 2006), but the mechanisms of retinal degeneration with RP are still unclear. For example, how mutations of some systemically expressed genes merely lead to photoreceptor degeneration remains elusive. It should be noted that more than half of RP patients are simplex (sporadic) or multiplex cases whose inheritance mode could not be clearly defined (Haim 1993; Jin *et al*. 2008). So far, no therapeutic approaches were successful in rescuing or even preventing retinal degeneration from further deterioration. Although supplements of vitamin A (Berson *et al*. 1993), lutein (Aleman *et al*. 2001) or docosahexaenoic acid (Hoffman *et al*. 2004) have been advocated by several investigators, their effects on RP are still controversial and the long-term benefits are unknown (Wlelber and Gregory-Evans 2006). This predicament is partly caused by the fact that the disease mechanisms have not been well elucidated. It is possible that RP patients with certain mutations obtain benefits from the above-mentioned supplementation (Wlelber and Gregory-Evans 2006). Thus, to consider treatment of RP patients, gene diagnosis is crucial for genetic classification and clinical trial of new drug or medicines. New biological therapies such as gene therapy (Ali *et al*. 2000), retinal cell transplantation (MacLaren *et al*. 2006), or growth factor treatment (Sieving *et al*. 2006), are developed for RP; although retinal cell replacement seems the most promising technique (Reh 2006).

Induced pluripotent stem cells

Pluripotent cells exist in the early embryo and can differentiate into all types of somatic cells. Since the establishment of human embryonic stem (ES) cells (Thomson *et al*. 1998), they are expected to be used for regenerative medicine. Many efforts have been made to directly convert somatic cells into pluripotent cells. For the first time Shinya Yamanaka's group successfully induced ES-like cells from mouse fibroblast cells in 2006 and named them 'induced pluripotent stem (iPS) cells' (Takahashi and Yamanaka 2006). After one year, the same group (Takahashi *et al*. 2007) and another group (Yu *et al*. 2007) successfully generated human iPS cells through direct programming of defined transcription factors, that are highly expressed in pluripotent cells. The iPS cells are shown to be very similar to ES cells in morphology, proliferation, differentiation capacity, genomic and epigenomic states (see review by Amabile and Meissner 2009).

From past two years, much progress has been made with regard to higher efficiency, integration-free strategy in the generation of iPS cells, and indeed, iPS cells of more animal species were generated. In the first report of human iPS cells, Takahashi *et al*. (2007) and his colleagues reported a relatively low efficiency of ES-like colonies. Recently, several groups have reported improved efficiency in generating human iPS cells with the supplementation of some additional factors including valproic acid (histone deacetylase inhibitor) and the SV40 large T antigen (Huangfu *et al*. 2008; Mali *et al*. 2008). Induced pluripotent stem cells were originally reprogrammed by the overexpression of four key transcription factors from introduced viral vectors (Takahashi *et al*. 2007); therefore, those genes were randomly integrated into the genome. There is no distinct integration site on the genome (Varas *et al*. 2009), indicating a safe utility of iPS, but there are still many concerns regarding exogenous integration of the genes, which may create artificial mutations and limit the utility of iPS cells. Subsequently, Yamanaka's group succeeded in designing a non-viral method in which the virus vectors were replaced by plasmids (Okita *et al*. 2008). More recently, iPS cells were generated directly by using non-integrating episomal vectors (Yu *et al*. 2009), piggyBac transposition (Woltjen *et al*. 2009), multiprotein expression vectors (Kaji *et al*. 2009), or chemicals (Lyssiotis *et al*. 2009). In addition, microRNA, a class of short single-stranded RNA molecules, has been shown to enhance the production of iPS cells (Judson *et al*. 2009). Recently, Kim *et al*. (2009) generated human iPS cells by direct delivery of reprogramming protein. Taken together, it is now possible to generate safer iPS cells by preferred non-invasive methods.

In vitro retinal differentiation

Induced pluripotent stem cells are ES-like pluripotent cells capable of differentiating into most, if not all, body cells (figure 1). This potential promises an unlimited source of differentiated cells to replace those lost in many human degenerative diseases. The *in vitro* differentiation of iPS cells would not only facilitate cell replacement therapy but could also be used in disease modelling or as a development research tool.

The process of *in vitro* differentiation to a somatic cell type always mimics its development *in vivo*, as does retinal differentiation from ES or iPS cells. Approximately at post-ovlatory day 25, the optic vesicle evaginates from the diencephalon of the neural tube of the human embryo and then folds to form the optic cup. After evagination of the lens vesicle, two layers of tissue form separately, including a RPE progenitor outer layer (Pax6+/Mitf+) and an inner presumptive neural retina layer (Pax6+/Rx+ neural retinal progenitors). Subsequently, the neural retinal progenitors produce different types of retinal cells, ganglion cells, horizontal

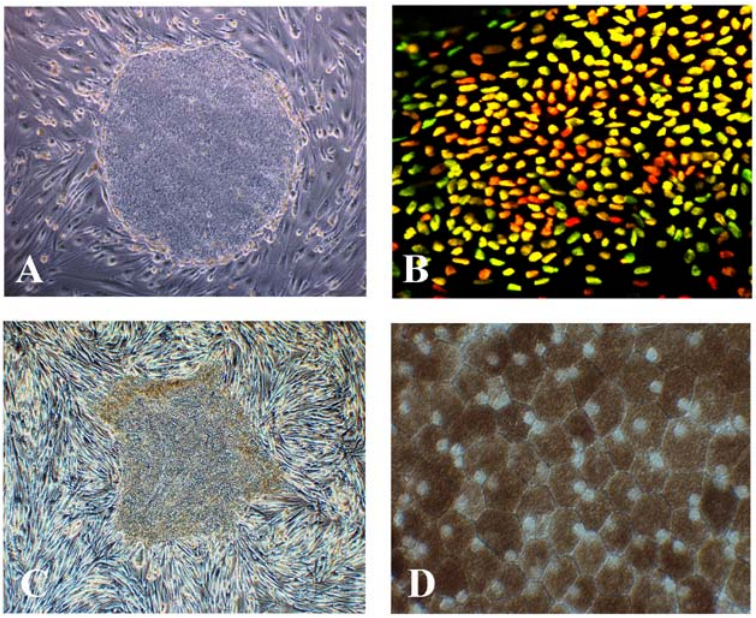

Figure 1. Human iPS cells (A) can differentiate into Pax6+ (green)/Mitf+ (red) RPE progenitor cells (B). *Cynomolgus* monkey derived iPS cells (C) can differentiate into pigmented, hexagonal RPE cells (D).

cells, cone photoreceptors (red/green opsin+ or blue opsin+), amacrine cells, Müller cells, bipolar cells and rod photoreceptors (rhodopsin+) in a stepwise style.

In the case of *in vitro* differentiation, RPE cells (figure 1) can be induced from ES cells under a PA6 co-culture condition (Kawasaki *et al*. 2002) or another defined culture method (Osakada *et al*. 2008a). Retinal ganglion cells can also be differentiated from ES cells (Jagatha *et al*. 2009). As retinal photoreceptor cells are the first-order neuron of retina and are always irreversibly lost in many of the retinal degenerative diseases, there is a great need for the generation of rod or cone photoreceptors from pluripotent cells. Since RPE and photoreceptors are initially developed from the same pool of ocular progenitor cells, these cells share a similar trajectory during cell differentiation.

There are several protocols reported for the differentiation of photoreceptors. For example, Reh's group reported that retinal progenitor cells can be efficiently generated from human ES cells (Lamba *et al*. 2006). Recently, the same group reported that generating retinal cells from human ES cells can restore the electroretinogram (ERG) response in Crx-knockout mice (Lamba *et al*. 2009). In their method, the ES colonies were cultured in floating conditions for three days with recombinant noggin, IGF-1 and Dkk-1, and then the colonies were replated on Matrigel-coated poly-D-lysine slide with a higher concentration of each of the factors. As a result, ~20% cells were Crx+, indicating the induction of photoreceptor precursors. Recently, our group successfully established a defined culture method that induces mouse and human ES cells to develop into both the photoreceptors and RPE cells (Osakada *et al*. 2008a). Hirami *et al*. (2009) in our group succeeded in producing mouse iPS and human iPS cells by using a similar strategy. In brief, we cultured human ES or human iPS colonies for 18 days using supplementation with nodal signalling inhibitor, Lefty-A and Dkk-1, which inhibits Wnt signalling (see review Osakada *et al*. 2008b). It should be noted that non-serum media was used in this study. After replating on poly-D-Lysine/laminin/fibronectin coated slide, Pax6+/Mitf+RPE progenitor cells and Pax6+/Rx+ retinal progenitors appeared approximately at day 30, and pigmented RPE cells emerged at day 40. Further, induction *in vitro* generated Crx+ retinal precursors at day 90 and rhodopsin+ or opsin+ photoreceptor cells at day 120. The induced RPE cells showed not only the hexagonal shape with pigments but also exhibited tight junctions, adherent junctions, microvilli and pigment granules through electron microscopic imaging (Osakada *et al*. 2008a). Further, these cells were capable of phagocytosis, indicating that they are functional RPE cells. Differentiated photoreceptor cells displayed a number of photoreceptor-specific markers; however, the physiological function of the photoreceptor cells was not evaluated. To assess the functionality of these cells, a cell electro-physiological approach or transplantation test may be useful. We recently established a novel method using small molecules instead of recombinant proteins to generate induced retinal cells from both human ES and iPS cells (Osakada *et al*. 2009). Thus, it is possible to generate human retinal neurons via chemical compounds. This would allow the differentiation of iPS cells under safer conditions, and free of serum and animal derivatives that may lead to cross-species contamination for cell replacement therapy.

Photoreceptor replacement

In the early stages of many retinal degenerative diseases, photoreceptor cells are damaged or lost, which leads to subsequent visual impairment. For such diseases, the replenishment of functional photoreceptors may be a good strategy for retinal regeneration, because gene therapy or growth factor supplement does not regenerate the photoreceptor cells lost to the disease. Indeed, substantial vision improvements were seen when a retinal cell suspension from human fetal retina was injected into the subretinal space of patients with RP or AMD (Das *et al*. 1999; del Cerro *et al*. 2000; Humayun *et al*. 2000). However, due to the limited source of fetal tissue and, most importantly, the ethical issues involved, this method is less practical for clinical application. Thus, it is important to find an alternative cell source for retinal cell replacement therapy and that alternative may be the *in vitro* culture of pluripotent cells in addition to differentiation technologies that would permit the generation of an unlimited cell source. In contrast to human ES cells, the use of human iPS cells does not pose a problem with ethical issues and may eliminate the risk of immunorejection with cell replacement therapies. However, donor cells and transplantation therapy should be carefully chosen as induced photoreceptor cells from RP patients (especially early onset RP) may die from causative mutations. Somatic mosaicism is a genetic state wherein different cell populations in one individual carry different genotypes. In the case of somatic mosaicism, it may be possible to generate healthy photoreceptors free of mutation; in fact, several RP cases of mosaicism have already been reported (Schwartz *et al*. 2003; Jin *et al*. 2007). In addition, for patients with identified mutation, *in vitro* gene correction of the iPS cells may be a preferable approach to obtain healthy retinal cells.

The optimal differentiation state of donor cells for functional integration into the host retina was determined by Ali's group in a landmark study for retinal cell transplantation. They defined the optimal ontogenetic stage of donor cells, which would allow successful rod photoreceptor transplantation (MacLaren *et al*. 2006). In addition, MacLaren and colleagues showed that the transplanted photoreceptor precursors (P3-6 post-mitotic rod precursors) can integrate, differentiate into rod photoreceptors, form synaptic connections and, most importantly, improve visual function. In addition to the donor cell source and transplantation technology, the host retina state is also crucial for retinal cell transplantation. Because the glial barrier of the diseased retina is suspected to prevent donor cell integration (Kinouchi *et al*. 2003), we disrupted the extracellular matrix by chondroitinases and matrix metalloproteases-2 (MMP2) in the diseased retina and found it to enhance the integration of transplanted photoreceptors (Suzuki *et al*. 2006, 2007). A recent study also showed that the outer limiting membrane (OLM) may be a physical barrier to photoreceptor integration following transplantation because disruption of this barrier has led to enhanced numbers of donor cells integrating into the outer nuclear layer (ONL) (West *et al*. 2008). Taken together, it is now technically feasible to transplant retinal cells differentiated from iPS cells.

RPE transplantation

Retinal pigment epithelial cells are physiologically essential for the maintenance of the neural retina; this includes outer segment shedding, supplying nutrients, and maintaining the blood-retinal barrier. Impairment in the RPE and photoreceptor dysfunction causes AMD. Several investigators have reported that the transplantation of autologous RPE increased the vision of AMD patients (Ma *et al*. 2009). However, this surgery has severe complications including the high risk of retinal hemorrhage. In contrast, we have demonstrated that the transplantation of ES-derived RPE cells restored visual function of the RCS rat (Haruta *et al*. 2004), a model of retinal degeneration primarily caused by RPE dysfunction. Another group also reported that transplantation of human ES-derived RPE cells rescued the retinal function in the RCS rat (Lund *et al*. 2006). It seems that ES-derived or iPS-derived RPE cell transplantation may enter into clinical trials soon. Compared to ES-derived cells, RPE cells induced from iPS cells may be a preferable source for RPE transplantation partly because RPE cells are reported to share some characteristics with antigen presenting cells (Rezai *et al*. 1997; Jorgensen *et al*. 2001). Thus, iPS cells may allow the development of autologous RPE, which should eliminate immunorejection. Furthermore, some investigators concluded HLA-haplotype banking of iPS cell lines may be a feasible strategy to match most precipitants (Nakatsuji *et al*. 2008). It should, however, be noted whether *in vitro* programming and differentiation would result in alterative immune state is unknown. Therefore, transplantation tests of allogenic RPE cells and autologous RPE are necessary before clinical translation to test whether immunorejection with the latter is indeed spared.

Patient-specific iPS cells: disease modelling

Induced pluripotent cells provide an exciting experimental platform to model human diseases. So far, iPS cells have been generated from patients with Parkinson's disease, Fanconi anaemia, thalassaemia, adenosine deaminase deficiency-related severe combined immunodeficiency, Shwachman-Bodian-Diamond syndrome, Gaucher disease type III, Duchenne and Becker muscular dystrophy, Huntington disease, juvenile-onset, type 1 diabetes mellitus, Down's syndrome/trisomy 21, and Lesch–Nyhan syndrome, amyotrophic lateral sclerosis (Dimos *et al*. 2008; Park *et al*. 2008; Raya *et al*. 2009; Ye *et al*. 2009). Most human inherited diseases are genetically and clinically heterogeneous. A large number of mutations in different genes may lead to a distinct

disease, while each mutation may represent a distinct phenotype. It is difficult to create animal models for all the reported mutations, but it is possible to have such iPS cells as disease models.

Most retinal degenerative diseases are complex and their mechanisms remain elusive; thus, appropriate therapies are difficult to establish. The etiology of AMD is still unclear although much progress has been made in the past five years. Currently, treatments for AMD include laser surgery, photodynamic therapy and anti-VEGF drug injection; however, each method is a stop-gap, which merely alleviates the symptoms of AMD by inhibiting neovascularization of the retina/choroid or by destroying the fragile, leaky blood vessels. Therefore, to exploit a novel treatment for AMD, it is crucial to determine its etiopathogenesis and to establish a proper model that mimics AMD. So far, genetically engineered animals, such as gene knockout mice, and laser-induced subretinal neovascularization models have been used for AMD research. However, these models have several weaknesses: (i) there is disparity in retinal structure and physiology due to the species difference; (ii) genetic manipulation or laser damage does not occur in AMD patients; and (iii) these models only reflect one side of the disease (e.g., drusen, neovascular) and do not reflect the overall manifestation or background status.

RP is not a unitary disease: it comprises a variety of inherited retinal disorders characterized by progressive night blindness and visual handicaps. So far, a number of animal models (e.g. mouse, rat, dog, pig and rabbit) have been established for RP modelling. Because RP is a well-defined genetic disease, each available animal model has an established genomic defect in an RP-related gene. All models can be classified into two groups: the retinal degeneration group in which the defect is caused by spontaneous mutation, e.g. the RCS rat; and the transgenic or knockout animal group in which the defective gene is designed through genetic engineering, e.g. the transgenic rabbit, pig, etc. Although photoreceptor degeneration does occur in these animals, gene knockouts or the extrinsic insertion of multi-copy genes do not exist in humans. Additionally, these animals have apparent differences in retinal architecture or cellulosity compared to humans. Because of these differences, the monkey should be a better model animal for RP; however, ethical issues and technical difficulties hinder its use as a model system.

The use of differentiated retinal neurons generated from a patient's own iPS cells may be a good choice for elucidating mechanisms of disease. In addition to their use with AMD and RP, induced retinal ganglion cells from iPS cells may be used as a research tool for glaucoma, the most prevalent disease worldwide that leads to blindness. By using patient's own retinal neurons as a research tool for elucidating disease mechanisms, we may discover important information such as the intrinsic factors which cause apoptosis in patients with retinal degenerative diseases. For many retinal degenerative diseases, details of apoptosis, e.g. how apoptosis is triggered are still largely unknown. Because apoptosis of retinal neurons is complex and involves many factors, iPS-derived retinal cells from patients may assist in determining how apoptosis is processed in a distinct genetic state.

Induced retinal neurons as a biological tool

Pluripotent cells provide a potential resource for biological research and drug screening. For example, ES cells have been used for pharmacological study (see review by Ho and Li 2006). Because the use of ES cells is constrained by ethical concerns and laws, it is impossible to generate patient-specific ES cell lines. The use of iPS cells overcomes this handicap and in fact, patient-specific iPS cell lines have already been created from patients with Parkinson's disease (Park *et al*. 2008), Fanconi anaemia (Raya *et al*. 2009), and sickle cell anaemia (Ye *et al*. 2009). Further, *in vitro* differentiated retinal cells from a patient's own iPS cells can be used as a biological tool for drug discovery in addition to toxicity screening. Because retinal cells from patients are not available for regular research, iPS-derived cells facilitate us to examine both the effectiveness and toxicity of a new drug under development or in clinical trials. As discussed above, some oral supplements, which are being used for RP, are still not accepted widely in the ophthalmology world because the mechanisms of efficacy are unknown and they have controversial trial results. We think iPS-derived photoreceptors or RPE cells are the optimal model for the pharmacological/physiological study of these drugs.

Challenges and perspectives

The use of iPS cells opens up a new avenue for biological science and regenerative medicine (figure 2); however, this new technology still faces many difficulties. First of all, the mechanism of nuclear reprogramming is a complex process, which has not been clearly mapped as yet. This makes it difficult to define the extent of reprogramming in the induced-cell lines. Further studies are required to address this issue. Secondly, the optimal iPS line is unresolved, because certain lines have shown differences in gene expression or epigenetic state; thus, the characterization of iPS lines is of critical importance. A guideline for defining the pluripotency of iPS cells has been established (Daley *et al*. 2009); however, further studies are required to establish a gold standard for line selection and to develop a high-throughput method for quality control. Additionally, current differentiation procedures are not sophisticated enough to guarantee efficiency and safety. For example, photoreceptor cells differentiated from iPS or ES cells are not high-performance in cell number. Although Reh's group efficiently generated retinal progenitor cells and photoreceptor precursors, the final photoreceptor cells seemed very rare (Lamba *et al*. 2006). The SFEB method can produce a higher percentage of rod photoreceptors, but *in vitro* induction requires more than four months for developing (Osakada *et al*. 2008a); thus, it would be

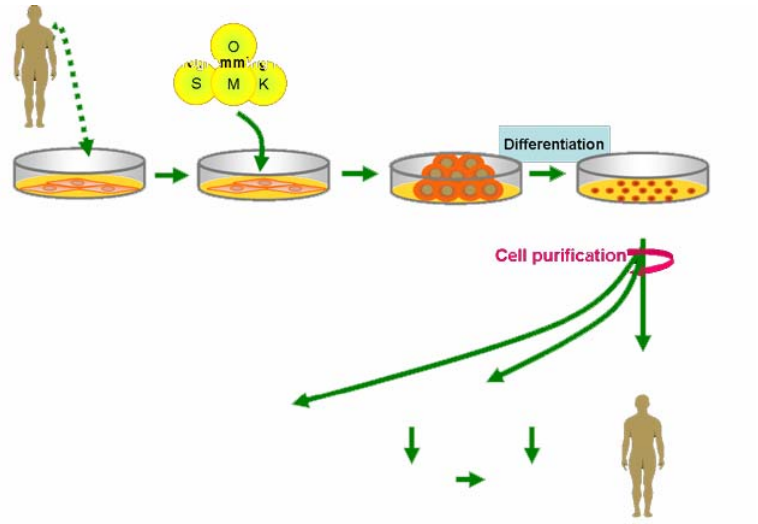

Figure 2. Illustration of patient-specific iPSC generation and its utility for disease research and cell replacement therapy.

beneficial to establish a new system to generate photoreceptor cells with higher efficiency in a shorter time. Another important issue of the *in vitro* differentiation technique is obtaining a pure cell population of retinal neurons through the elimination of pluripotent cells and refining differentiated retinal cells. Identification of suitable cell type specific surface antigens for efficient sorting of desired cell types is required. Additionally, for the purpose of cell transplantation, the *in vitro* differentiation strategy should avoid any animal derivatives which have a risk of cross-contamination. On the other hand, to model inherited retinal degenerations, it is essential to identify genetic mutations by gene diagnosis. Therefore, identifying pathological mutations for all types of patients is of particular importance. Currently, iPS cell generation, maintenance, and differentiation have a high-cost and are technically difficult, hence, developing chemically defined methods of iPS operation and differentiation may reduce the costs and increase safety.

Induced pluripotent stem cell lines have been produced from many species, including humans and provide us with a new tool for disease study. For example, porcine iPS cells (Ezashi *et al*. 2009; Wu *et al*. 2009) may be useful for the study of retinal degeneration or preclinical trials because the rhodopsin transgenic pig that models RP has been generated (Li *et al*. 1998). Recently, a transgenic model for retinal degeneration in the rabbit has been generated (Kondo *et al*. 2009). It is expected to create rabbit iPS cells for cell transplantation in this model. Rhesus monkey iPS cells have also been successfully generated (Liu *et al*. 2008). Additionally, we have generated iPS cell lines from cynomolgus monkey fibroblasts and have already differentiated them into retinal cells (figure 1) (Okamoto and Takahashi 2009). A recent study reported that human ES-derived retinal cells can migrate and integrate with mouse retina and lead to the recovery of retinal function (Lamba *et al*. 2009). For the clinical use of cell replacement therapy, the possibility of immunologic rejection must be considered and cleared. To address this issue, it is necessary to test autologous transplantation with a control of allogenic transplant using animals (e.g., monkey) iPS cells. In addition, current cell transplantation technique still does not guarantee functional recovery. For instance, in addition to suspension cell transplantation, using a sheet of RPE cells or photoreceptor cells may be a promising method to enhance the transplantation efficiency (Redenti *et al*. 2009). Another challenge for cell transplantation is to elucidate how the host retina prevents donor cell integration. These barriers may include Müller cells (Kinouchi *et al*. 2003) or microglia (Suzuki *et al*. 2006); however, further studies are required to overcome these barriers and improve the efficiency.

Direct reprogramming of human somatic cells provides a new opportunity to study retinal degenerative diseases and can become a cell source for retinal regeneration. While the clinical utility of iPS cells will depend on the efficiency, safety, and cost-effectiveness, more studies are required to determine the full potential of iPS technology. With growing advancements in this field, we can certainly look forward to tremendous advancements in both the basic science and therapeutics for retinal degenerative diseases.

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