·Review·

Research on induced pluripotent stem cells and the application in ocular tissues

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Received: 2014-03-11 Accepted: 2015-02-02

Abstract

• **Induced pluripotent stem cells (iPSCs) were firstly induced from mouse fibroblasts since 2006, and then the research on iPSCs had made great progress in the following years. iPSCs were established from different somatic cells through DNA, RNA, protein or small molecule pathways and transduction vehicles. With continuous improvement of technology on reprogramming, the induction of iPSCs became more secure and effective, and showed enormous promise for clinical applications. We reviewed different reprogramming of somatic cells, four kinds of pathways of reprogramming and three types of transduction vehicles, and discuss the research of iPSCs in ophthalmology and the prospect of iPSCs applications.**

• **KEYWORDS:** induced pluripotent stem cells; reprogramming; ocular tissues

DOI:10.3980/j.issn.2222-3959.2015.04.31

Guo XL, Chen JS. Research on induced pluripotent stem cells and the application in ocular tissues. *Int J Ophthalmol* 2015;8(4):818-825

INTRODUCTION

Stem cells have the capacity to self-renew and differentiate into other cell types. Human embryonic stem cells (hESCs) are inner cell masses of the developing embryo. hESCs have the potential to differentiate into cells from the three germ layers: ectoderm, mesoderm and endoderm. Consequently, hESCs are described as pluripotent. However, ethical concerns regarding hESCs from the inner cell masses of developing blastocysts led to a drive to investigate alternative methods of deriving pluripotent stem cells. Induced pluripotent stem cells (iPSCs) are reprogrammed from somatic cells and can be theoretically differentiated into all kinds of cell types, making them "rejuvenate" back to the state of ESCs. iPSCs represent a significant advance towards the applications of stem cells in regenerative medicine. Moreover, they can also be used to address the problems of immune rejection and ethical issues for the clinic. The clinical translation of basic researches to treatments has gotten more and more attention in the worldwide. iPSCs have generated much interest and research into their potential in restoring vision. There are several advantageous properties for ocular regenerative approaches, which include ocular immune privilege and accessibility. So there will be a bright future of iPSCs applications in ophthalmology and other clinical subjects. Advances in our understanding of iPSCs and their application ocular tissues will enable us to overcome current clinical obstacles [1]. This article addresses the current status of knowledge concerning the generation of iPSCs *in vitro*, iPSCs reprogramming *in vivo*, reprogramming ocular cells into iPSCs and differentiation of iPSCs into ocular cells. It will highlight the application of iPSCs in ocular tissue cells to date and their potential for future clinical use.

RESEARCH ON INDUCED PLURIPOTENT STEM CELLS

Induced Pluripotent Stem Cells Based on Diverse Target Cells Takahashi and Yamanaka [2] firstly transduced four defined transcription factors (Oct4, Klf4, Sox2 and c-Myc, known as 4TFs) into mouse skin fibroblasts, and got pluripotent stem cells like ESCs, then named them "iPSCs". Soon, iPSCs had been induced from human fibroblasts, which shed light onto the application of iPSCs for clinical therapy. Since cells source for iPSCs generation involved the three germ layers, all cell types of an adult organism could be theoretically induced into iPSCs, and the list of cells for reprogramming had been greatly expanded. Dermal fibroblasts could be obtained from skin biopsy, and then be expanded for 5-8 passages in order to achieve enough cell numbers for iPSCs generation. The invasiveness of such manipulations could be overcame by the derivation of iPSCs from patient blood samples or even urine [3,4]. Furthermore, iPSCs could be generated from patients with genetic disease

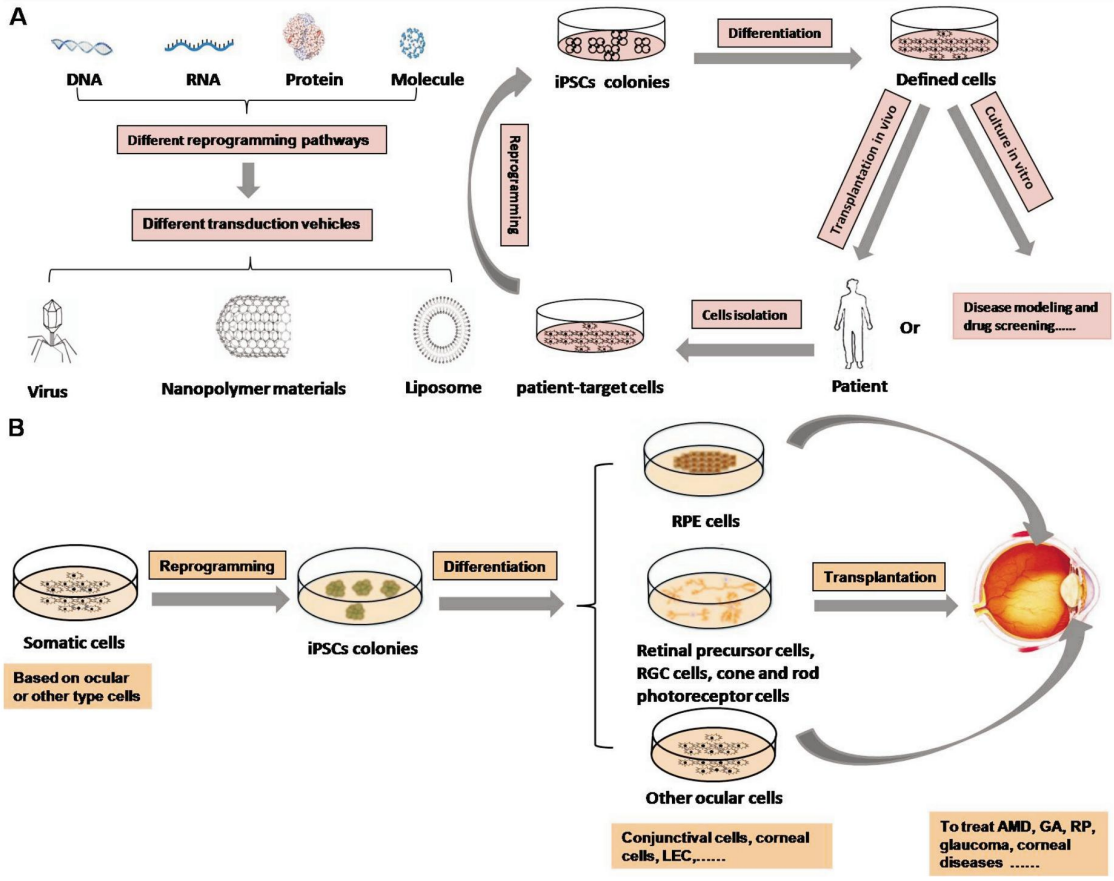

Figure 1 The reprogramming and envisioned application of iPSCs A: Scheme to generate patient-specific iPSCs for transplantation, disease model, drug screening and so on; B: Scheme to differentiate iPSCs reprogrammed from somatic cells into ocular cells for transplantation.

or cancer, and then be used in the study of disease mechanisms (Figure 1A). Cancer-derived iPSCs could be served as a useful tool to understand the effectiveness of cancer drugs and to explore the molecular mechanisms in cancer development [5]. Some kinds of cancers may be stem cell-driven following a similar reprogramming process like iPSCs[6].

Reprogramming Induced Pluripotent Stem Cells Through Four Kinds of Pathways Mouse and human cells could be reprogrammed into iPSCs by a series of TFs *via* DNA pathway (Table 1). iPSCs were firstly generated from mouse fibroblasts by transducing 4TFs. Human cells could also be reprogrammed by the same set of 4TFs. In 2008, Duinsbergen *et al*[7] and Wernig *et al*[8] reprogrammed somatic cells into iPSCs by 3TFs (Oct4, c-Myc, Klf4; Oct4, Sox2, Klf4). Then Kim *et al*[9] implemented neural stem cells reprogramming with 2TFs (Oct4, Klf4 or c-Myc), while Huangfu *et al* [10] used another 2TFs (Oct4, Sox2) for reprogramming. Surprisingly, Kim *et al* [11] used only 1TFs for generation of iPSCs from neural stem cells (Oct4).

However, virus-mediated procedure of DNA pathway had potentially harmful effect, and affected the clinical use of iPSCs. With ongoing research, people had paid attention to the reprogramming with non-genetic approach. Biologists reprogrammed cells into iPSCs with RNA, proteins, and small molecules, which made great advance in the safety and efficiency (Figure 1A). RNA pathway became more diverse and effective [12]. Yakubov *et al* [13] reprogrammed iPSCs through RNA pathway. Warren *et al* [14] achieved the reprogrammed iPSCs from human body cells by mRNA. Through transduction mRNA synthesis of 4TFs *in vitro* and conduct glycosylation and methylation modification in the 5' end, the half-life of mRNA was extended. Therefore, the effect of transfection could be maintained longer. Anokye-Danso *et al* [12] cloned exogenous miR302/367 genes to pLOVE vector, which was transfected into human and mouse somatic cells plus Hdac2, and successfully reprogrammed them into iPSCs.

Through protein pathway, reprogramming of iPSCs became more safe for future clinical applications. More recently, recombinant protein transductions (RPTs; Klf4, Oct4, Sox2, and c-Myc); could be used to produce iPSCs not only from prokaryotic expression vectors but also from eukaryotic expression vectors [15-17]. The based-proteins of human iPSCs could be used to generate functional dopamine neurons. Transplantation of these neurons into rat model of Parkinson's disease significantly compensated the motor disturbance [18], which opened a new path for human individual treatment of Parkinson's disease without the integration of exogenous genes.

Table 1 Summary of transcription factors of reprogramming for iPSCs

Reprogramming transcription factors	Cell type	Reference
Oct4, Sox2,c-Myc and Klf4	Embryonic and adult fibroblasts	Takahashi and Yamanaka, 2006; (PMID:16904174)
Oct4, Sox2,c-Myc and Klf4	Fibroblasts	Takahashi, 2007; (PMID:18079707)
Oct4, Sox2,c-Myc and Klf4	Keratinocytes	Aasen, 2008; (PMID:18931654)
Oct4, Sox2,c-Myc and Klf4	Multiple somatic tissues	Wernig, 2008; (PMID:18594521)
Oct4, Sox2,c-Myc and Klf4	Blymphocytes	Hanna, 2008; (PMID:18423197)
Oct4, Sox2,c-Myc and Klf4	Embryonic fibroblasts	Okita, 2008; (PMID:18845712)
Oct4, Sox2,c-Myc and Klf4	Hepatocytes and fibroblasts	Stadtfeld, 2008; (PMID:18818365)
Oct4, Sox2,c-Myc and Klf4	Blood cells	Loh, 2009; (PMID:19299331)
Oct4, Sox2,c-Myc and Klf4+2A peptide	Fibroblasts	Kaji, 2009; (PMID:19252477)
Oct4, Sox2,Nanog and lin28	Fibroblasts	Ebert, 2009; (PMID:19098894)
Oct3/4, Sox2,c-Myc and Klf4	Fibroblasts	Nakagawa, 2008; (PMID:18059259)
Oct4, c-Myc and Klf4	Neural stem cells	Duinsbergen, 2008; (PMID:18656469)
Oct4, Sox2 and Klf4	Fibroblasts	Wernig, 2008; (PMID:18371415)
Oct4, Sox2 and Esnb	Embryonic fibroblasts	Feng, 2009; (PMID:19136965)
Oct4 and Klf4 or c-Myc	Neural stem cells	Kim, 2008; (PMID:18594515)
Oct4 and Sox2	Fibroblasts	Huangfu, 2008; (PMID:18849973)
Oct4	Neural stem cells	Kim, 2009; (PMID:19203577)
Oct4	Keratinocytes	Grinnell, 2007; (PMID:16932739)

As the reprogramming was mainly involved in chromatin remodeling across acetylation or demethylation and some key signaling pathways (MAPK, Wnt, TGF-β, MEK-ERK, sonic hedgehog, *etc*), iPSCs reprogramming could be achieved by intervention in the epigenetics and key signaling pathways. For example, mouse fibroblasts could be reprogramming into iPSCs through the activation of sonic hedgehog signaling (by Shh, purmorphamine, or oxysterol) in combination with only 1 TF (Oct4). Li *et al* [19] also reported that somatic cells could be reprogrammed into iPSCs by Oct4 and four kinds of small molecules (VPA, CHIR99021, 616452, tranylcypromine), which replaced the remaining transcription factors (Sox2, Klf4, and c-Myc). Recently, Hou *et al* [20] reported that the 4TFs could be replaced by six small molecules [VPA, CHIR99021, 616452, tranylcypromine, Forskolin (FSK), deazaneplanocin A (DZNep)] to achieve iPSCs reprogramming through small molecule pathway based on the research for inhibitors or activators affecting the epigenetic and other crucial signaling pathways. Moreover, DZNep played a crucial role in promoting the expression of endogenous Oct4.

Transduction Vehicles of Reprogramming Induced Pluripotent Stem Cells

Virus transduction Takahashi and Yamanaka [2] firstly generated iPSCs with transduction of 4TFs into mouse somatic cells by retrovirus. Stadtfeld *et al* [21] transduced 4TFs into mouse somatic cells by adenovirus. Similarly, Hanna *et al* [22] used lentivirus to import 4TFs into rat B lymphocytes. Furthermore, Fusaki *et al* [23] achieved the reprogramming of human somatic cells by means of Sendai virus (RNA virus), which mediated the reprogramming without gene insertion, and therefore ensured the safety in use.

Liposome and nanopolymer materials transduction Park *et al* [24] found that safety of reprogramming iPSCs could be greatly improved through non-virus transduction of two kinds of recombinant vectors (pCX-OKS-2A, pCX-c-Myc) into mouse embryonic fibroblast (MEF) cells with liposome transfection reagents. However, the efficiency of liposome transduction was also reduced when comparing with the virus transduction. Montserrat *et al* [25] transfected large pCAG-OSKMG plasmid (11 kb) fused 4TFs into human fibroblasts by using three different end-modified poly-β-amino esters as non-viral delivery system, and generated iPSCs. They revealed that poly-β-amino esters could be used to deliver a single polycistronic reprogramming vector into human somatic cells and achieved higher transfection efficiency than conventional transfection reagents.

Research of Reprogramming *in Vivo* The above studies mainly concern the reprogramming of somatic cells *in vitro*. Recently, iPSCs reprogramming *in vivo* had been reported. Abad *et al* [26] showed that transitory induction of 4TFs expression in reprogrammable mice resulted in teratomas emergence from multiple organs, implying that reprogramming could occur *in vivo*. *In vivo*, iPSCs had an unprecedented capacity to form embryo-like structures and represented a more primitive, potential, and plastic state than ESCs or *in vitro* iPSCs. This research displayed that tissue microenvironment *in vivo* might provide cues and mechanical forces to promote reprogramming. Although the

concept of *in vivo* reprogramming on pluripotency was still in its youth, at its infancy, it could be of great use in regenerative medicine and help overcome the disadvantages of iPSCs generated *in vitro* [3].

APPLICATION OF INDUCED PLURIPOTENT STEM CELLS IN OCULAR TISSUES

The study of iPSCs had also made a remarkable impact on the field of ophthalmology. iPSCs not only could be reprogrammed from ocular cells, but also differentiated into ocular cells for ocular disease modeling and transplantation therapy (Figure 1B).

Reprogramming Ocular Cells into Induced Pluripotent Stem Cells A large population of ocular tissue cells had been examined for their reprogramming potential. Balasubramanian *et al* [27] reprogrammed corneal limbal epithelial cells of adult rat eye into iPSCs by means of recruitment the endogenous genes without introduction of genetic materials and exogenous factors. Such non cell-autonomous reprogramming iPSCs were capable to differentiate into functional neurons, cardiomyocytes, and hepatocytes, which might facilitate autologous cell therapy and be more suitable for potential clinical applications. Subsequently, Chien *et al* [28] successfully reprogrammed human corneal keratocytes into iPSCs, and demonstrated that human keratocyte-derived iPSCs, when combined with carboxymethyl-hexanoyl chitosan (CHC) hydrogel, could be used as a rapid delivery system to efficiently enhance corneal wound healing *in vivo*. Yang *et al* [29] demonstrated the feasibility of generating iPSCs from the mouse conjunctiva. Conjunctival cells were readily obtained during the course of many routine conjunctival biopsies and ophthalmic procedures. Therefore, conjunctiva could be another reliable source of iPSCs. Deng *et al* [30] presented a simple and practical method for generation patient-tailored iPSCs from human Tenon's capsule fibroblasts (HTFs). These cells would serve as a valuable and preferable candidate donor cells for ophthalmological regenerative medicine. Qiu *et al* [31] described that iPSCs were generated from human lens epithelial cells (HLECs) of cataract patients. HLECs-derived iPSCs could be efficiently guided to differentiate into lens cells, which exhibited reduced expression of epithelial mesenchymal transition (EMT) markers compared with human ESCs and fibroblast-derived iPSCs. Recently, ocular ciliary body epithelial cells had been reprogrammed with high reprogramming efficiency. Oct4 alone was sufficient to reprogram ciliary body epithelial cells into iPSCs through sphere formation. So ciliary body epithelial cells could be an exciting candidate for cellular reprogramming strategies [32-34]. As we know, iPSCs usually harbor residual DNA methylation signatures characteristic of their somatic tissue of origin, which favors their differentiation along lineages related to the donor cells, while restricting alternative cell fates [35,36]. Therefore, ocular cells-derived iPSC lines should be more suitable for ophthalmological transplantation or eye disease modeling than other iPSC lines[30].

Differentiation of Induced Pluripotent Stem Cells into Ocular Cells iPSCs had a wider differentiative potential than somatic stem cells, providing an unlimited source of ocular cells for the treatment of ocular diseases. iPSCs-derived ocular cells could offer opportunity to study the molecular and cellular mechanisms underlying ocular tissue development, and the establishment of *in vitro* models of human ocular degenerative diseases. iPSCs could be differentiated into corneal epithelial cells (CECs) by co-culture with limbal fibroblasts (LF) or PA6 stromal cells. Such co-culture treatment could replicate corneal epithelial stem cell niche, produce stromal cell-derived inducing activity (SDIA), and initiate Pax6, P63, K3 and K12 expressions [37,38]. Recently, Mikhailova *et al* [39] showed that two small-molecule inhibitors, SB-505124 (TGF-β inhibitor) and IWP-2 (Wnt inhibitor), in combination with basic fibroblast growth factor (bFGF) in serum-free and feeder-free conditions, differentiated human iPSCs toward eye precursors and further toward CECs. iPSCs could be differentiated to trabecular meshwork (TM)-like cells. When these iPSCs derived TM-like cells were transplanted into saponin treated anterior segments, they were able to fully restore intraocular pressure homeostatic function. This is thus a major conceptual step toward developing iPSCs therapy for open-angle glaucoma [40]. iPSCs application in retinal differentiation and treatment for blindness caused by retinal disease had already made great achievements. Okamoto and Takahashi[41] firstly induced RPE from monkey iPSCs. iPSCs derived retinal pigment epithelium (iPSCs-RPE) could be used for autologous or allogeneic transplantation to test the possibility of immune rejection and to evaluate their function *in vivo*. It was reported that addition of factors related to RPE development at specific times induced the conversion of approximate 80% human iPSCs into RPE phenotypes in only 14d [42]. iPSCs-RPE constitutively expressed TGF-β and suppressed activation of T cells *via* soluble TGF-β [43]. iPSCs-RPE could be induced with easier and less labor-intensive generation and displayed normal pigmentation and morphology. They were also capable of expression of specific cell markers, polarization of cell membrane, secretion of vascular endothelial growth factor, and phagocytic activity. Transplantation of such iPSCs-RPE into the subretinal space of mice displayed the presence of numerous pigmented clusters and rhodopsin-positive fragments at one week after injection, suggesting the capacity

of survival and phagocytosis of iPSC-RPE cells *in vivo* [44]. Li *et al* [45] provided the direct evidence of functional recovery in a clinically relevant model of retinal degeneration using iPSCs transplantation and supported the feasibility of autologous iPSCs transplantation for retinal and macular degeneration featuring RPE loss. Human iPSCs-RPE were injected into the subretinal space of model mice of retinitis pigmentosa (RP), with the result that none of the transplanted mice developed tumors and electroretinogram demonstrated improved visual function. The first clinical study using human iPSC-RPE cells started to recruit patients in August 2013 [46,47] and began with cell transplantation procedures within 2014[3,48]. They had been working on the generation of RPE sheets from patient-specific iPSCs for subretinal transplantation in age-related macular degeneration (AMD), hoping that the transplanted iPSCs-RPE would grow and repair the affected RPE. The primary goal of this study was to assess the safety concerning over medical use of the cells derived from iPSCs in human.

iPSCs-neuroretinal (iPSCs-NR) cells could be obtained by mimicking the order and time course of retinogenesis [49]. Tucker *et al* [50] designed adult mouse iPSCs to produce retinal precursors and subsequent photoreceptor cells for retinal transplantation to restore retinal function in degenerative hosts. Parameswaran *et al* [51] also demonstrated that iPSCs represented a renewable and robust source of retinal progenitor cells (RPCs), and were capable of generating a wide range of retinal cell types that included retinal ganglion cells (RGCs), cone, and rod photoreceptors.

iPSCs could be differentiated into retinal cells of multilayers by different methods. Du *et al* [52] discussed that RPE and photoreceptors derived from patient-specific iPSCs could serve as a valuable tool in elucidating the mechanism of pathogenesis and drug discovery for AMD patients. Recent work had shown that the multilayered retinal tissues such as optic cup or optic vesicle (OV) could be formed by self-organization *in vitro* from a homogeneous population of ESCs and iPSCs, which were subsequently suitable for study in retinal development and disease treatment [53]. OV-like structures from iPSCs of patient with gyrate atrophy (GA) due to an A226V mutation in ornithine-δ-aminotransferase (OAT) could be used to test pharmacologic and genetic treatment approaches. OAT activity was restored in the gene-corrected iPSCs-RPE derived from OV-like structures of GA patients[54]. Phillips *et al* [55] demonstrated that cultured T-lymphocyte-derived iPSCs (TiPSCs) could differentiate into OV and had the capacity to self-assemble into rudimentary NR structures. The indicative markers of chemical and electrical synapses were expressed. The spontaneous formation of a self-organized NR from iPSCs cultured together with ECM was sufficient to induce a rapid conversion into RPCs in 5d. These RPCs had the ability to differentiate efficiently into Crx^+ photoreceptor precursors (Crx is one of the earliest known photoreceptor markers) after 10d, and subsequently acquired rod photoreceptor identity within four weeks[56]. Reichman *et al* [57] developed a simple retinal differentiation method, based on confluent iPSCs. Only in 2wk both RPE and self-forming NR-like structures containing RPCs could be generated. The sequential differentiation from RPCs to seven types of neuroretinal cells in maturated NR-like structures as floating cultures were reported. Furthermore, Notch pathway inhibition boosted the generation of photoreceptor precursor cells.

Sanges *et al* [58] showed the increase in thickness of the inner nuclear layer and regeneration of retinal ganglion cell layer in N-methyl-D-aspartate (NMDA)-damaged retina at one month after 6-bromoindirubin-3'-oxime (BIO) and hematopoietic stem and progenitor cells (HSPCs) transplantation. BIO mediated Wnt activation. The study demonstrated that retinal damage was essential for cell-hybrid formation *in vivo*. The retinal neurons could be reprogrammed back to precursor cells *in vivo* after fusion of mouse retinal neurons with HSPCs and activation of Wnt signaling. Most recently, an initial report had provided evidence for the *in vivo* reprogramming of one type of retinal neurons into another, suggesting that the reprogramming of postmitotic photoreceptor neurons might even prevent retinal degeneration. The generation of neurons from cells of patients with retinal disorders might provide novel insights into disease pathomechanisms, disease progression, early diagnosis, drug discovery, and therapy validation[59,60].

APPLICATION AND PERSPECTIVE

The reprogramming of somatic cells into pluripotent stem cells or other cell types showed promising use in treatment of intractable degenerative diseases, such as direct reprogramming for heart repair, corneal repair, restoration of retinal function, remedy of Parkinson's disease, and treatment of sickle cell anemia *etc* [17,28,44,61,62]. Especially, the reprogramming *in vivo* happens in the physiological microenvironment of human body, which could be-higher efficiency and greater safety. In addition, the reprogrammed cells can be served as a tool for study of diseases and research on drugs and genes. Before the practical use of iPSCs in clinical situations, there is still a long way to go. Safe methods for iPSCs generation and high efficacy for reprogramming are of the utmost importance. The negative selection of pluripotent cells or positive selection of differentiated cells holds the key point to minimize the risk of tumor formation. Integration-free and xeno-free methods for

iPSCs are recommended for ensured safety, but it is necessary to find a way to efficiently and precisely modify the pathological gene expressions in hiPSCs from the patients. That is to say, the pathogenic mutation needs to be corrected first before the transplantation of autologous iPSCs-derived cells to treat the genetic-based retinal disorders [63,64]. iPSCs and iPSC-derived ocular tissue cells could be used to interrogate disease pathophysiology, develop drug, genome editing, gene augmentation and cell-based therapies for ophthalmology[65].

With the renaissance of iPSCs, the use of stem cells in regenerative medicine has made a significant advancement, and the problems of ethical issues and immune rejection are eliminated. Cell replacement from iPSCs may provide a viable treatment option for some severe retinal degenerative disorders such as AMD and RP. Although the efficiency and safety of reprogramming have been improved greatly, there is still a long way to go for applied the clinical application of iPSCs. With the constant improvement of reprogramming technology and approaches, iPSCs will be widely used in stem cell treatments. The groundwork for iPSCs-based ocular therapies has already been laid, offering guarded hope to individuals suffering from presently untreatable blinding diseases [66].

ACKNOWLEDGEMENTS

Foundations: Supported by the National Natural Science Foundation of China (No. 81371689); Natural Science Foundation of Guangdong Province (S2013010013391).

Conflicts of Interest: Guo XL, None; **Chen JS,** None.

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