• Review Article •

Differentiation of retinal ganglion cells from induced pluripotent stem cells: a review

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Abstract

• Glaucoma is a common optic neuropathy that is characterized by the progressive degeneration of axons and the loss of retinal ganglion cells (RGCs). Glaucoma is one of the leading causes of irreversible blindness worldwide. Current glaucoma treatments only slow the progression of RGCs loss. Induced pluripotent stem cells (iPSCs) are capable of differentiating into all three germ layer cell lineages. iPSCs can be patient-specific, making iPSC-derived RGCs a promising candidate for cell replacement. In this review, we focus on discussing the detailed approaches used to differentiate iPSCs into RGCs.

• KEYWORDS: glaucoma; retinal ganglion cells; induced pluripotent stem cells; differentiation

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INTRODUCTION

Glaucoma is a common optic neuropathy and one of the leading causes of irreversible blindness in the world. It is characterized by the progressive degeneration of axons and the loss of retinal ganglion cells (RGCs). Approximately 111.8 million people will be affected by glaucoma by 2040[1]. The main risk factor for glaucomatous optic neuropathy is high intraocular pressure (IOP). Current glaucoma treatments that primarily target increased IOP, including topical eyedrops, laser treatment, and surgeries, but only slow the progression of RGC loss[2]. There are an increasing number of recent reports on the development of neuroprotective therapies for glaucoma, that could be used as adjunctive treatments to lower IOP[3]. These studies investigated neuroprotective strategies, including the delivery of a neurotrophic factor, the molecular application of anti-apoptosis and anti-inflammation treatments, and the reduction of oxidative stress[4]. However, the clinical application is partially limited, because long-term maintenance of the supplementation is difficult and there is a possibility of compulsory repeated interventions. Recently, cell-based therapy has been demonstrated to be effective for the treatment of several diseases[5-8]. The cells used for these therapies include mesenchymal stem cells (MSCs), dental pulp stem cells, embryonic stem cells (ESCs), and induced pluripotent stem cells (iPSCs) and so on. In 2006, Takahashi and Yamanaka[9] successfully reprogrammed mouse and adult fibroblasts to a pluripotent state by using four transcription factors (*Oct3/4, Sox2, Klf4,* and *c-Myc*) that are involved in the pluripotency maintenance in ESCs. The resulting cells, iPSCs, could form colonies that are morphologically similar to ESCs and are capable of differentiating into all three germ layer cell lineages. Since iPSCs could be reprogrammed by the somatic cells of a patient, they could maintain the total unique genomic information of each individual. These patient-derived iPSCs could serve as a perfect *in vitro* model for genetic disease studies and thus have a promising role in the development of personalized treatment. These factors have resulted in an explosion of studies that attempt to exploit the reprogramming of somatic cells into iPSCs, reporting various modified protocols designed to improve the reprogramming efficiency and facilitate clinical application. For example, instead of retroviruses, multiple studies have used plasmid[10-11], miRNA[12], and protein[13] as transcript factors delivery vectors to prevent the risk of insertional mutagenesis of the host cells. Other reports indicate that the addition of small molecules, such as valproic acid (VPA)[14], AZA5-aza-cytidine (AZA)[15], butyrate[16], vitamin C[17], transforming growth factor-β (TGF-β) receptor inhibitor (A-83-01)[18-19], MEK inhibitor (PD325901)[18-19], GSK3β inhibitor (CHIR99021)[18-19], and ROCK inhibitor (HA-100)[18-19] could enhance reprogramming efficiency and even replace the use of certain transcription factors in iPSCs generation protocols. Table 1 shows several examples of the experimental features of protocols to transform somatic cells into iPSCs. Insight is required regarding how to induce iPSCs to differentiate into the specialized cell fate of interest.

Table 1 Examples of experimental features from somatic cell to iPSCs

Cell source	Culture condition	Reprogramming factors	Delivery method	Time length (d)	Efficiency (%)	Reference
Mouse fibroblasts	Cell feeder	Oct3/4, Sox2, C-myc, Klf4	Retrovirus	N/A	0.02	[9]
Human fibroblasts	Cell feeder	Oct4, Sox2, Nanog, Lin28	Lentivirus	15	0.022	[22]
Human fibroblasts	Cell feeder	Oct3/4, Sox2, Klf4, Sall4	Retrovirus	25	0.014	[23]
Human blood mononuclear cells	Cell feeder	Oct4, Sox2, Klf4, C-myc, Lin28	Plasmid	14	0.05	[11]
Human fibroblasts	Cell feeder	Oct3/4, Sox2, Klf4, Glis1	Retrovirus	22	0.17	[24]
Human fibroblasts	Cell feeder	Oct3/4, Sox2, Klf4, L-myc, Lin28, P53	Plasmid, shRNA	14	0.03	[25]
Human keratinocytes	Cell feeder	Oct4, Sox2, Klf4, C-myc, Rem2	Retrovirus	12	0.2	[26]
Human fibroblasts	Cell feeder	Oct4, Sox2, Myc, Klf4, Sv40 large T antigen	Lentivirus	14	0.46	[27]
Human fibroblasts	Cell feeder	Oct3/4, Sox2, C-myc, Klf4	Adenovirus	25	0.0002	[28]
Mouse and human fibroblasts	Cell feeder	Oct3/4, Sox2, C-myc, Klf4	2A peptide	9	2.5	[13]
Human fibroblasts	Cell feeder	Oct3/4, Sox2, C-myc, Klf4	PiggyBac transposon	14	0.01	[13]
Human fibroblasts	Cell feeder	Oct3/4, Sox2, C-myc, Klf4	PiggyBac transposon	10-14	0.03	[29]
Human adipose stromal cells	Cell feeder	Oct4, Sox2, Nanog, Lin28	Minicircle DNA	18	0.0075	[30]
Human fibroblasts	Xeno-free, feeder-free	Oct3/4, Sox2, Klf4, L-myc, Lin28, TP53, A-83-01, sodium butyrate, CHIR99021	Plasmids, shRNA, small molecules	16	0.1	[10]
Mouse fibroblasts	Cell feeder	Mir-200c, Mir-302s, Mir-369s	MiRNA	15	0.1	[12]
Human fibroblasts	Cell feeder	Mir-200c, Mir-302s, Mir-369s	MiRNA	20	0.002	[12]
Human fibroblasts	Cell feeder	Oct4, Sox2, VPA	Retrovirus, small molecules	N/A	0.01	[31]
Human fibroblasts	Cell feeder	Oct4, Sox2, Klf4, VPA	Retrovirus, small molecules	N/A	1	[31]
Human bone mesenchymal stromal cell	Feeder-free	Oct4, Sox2, Nanog, Lin28, Sv40lt, Klf4, C-myc, A-83-01, PD325901, CHIR99021, HA-100	Plasmids, small molecules	N/A	N/A	[18]
Mouse fibroblasts	Cell feeder	Oct4, tranylcypromine, VPA, CHIR99021, 616452	Lentivirus, small molecules	18d	0.03	[32]
Human fibroblasts	Xeno-free, feeder-free	Oct3/4, Sox2, C-myc, Klf4	Lentivirus	7-14d	0.3	[33]

iPSCs: Induced pluripotent stem cells; VPA: Valproic acid.

An increasing number of reports have indicated that iPSCs could be differentiated into RGCs, photoreceptors, and retinal pigment epithelium (RPE) under appropriate conditions[20-21]. The current review provides a perspective on the key methods that led to the differentiation of RGCs, and divulged the problems that must be solved before the iPSCs-derived RGCs could fulfill its potential in medical applications, such as the mechanisms of pathology, screening treatment drugs, and development of cell-based and patient-specific therapies targeting glaucoma and other optic neuropathies.

FACTORS INVOLVED IN THE DIFFERENTIATION OF RGCS FROM IPSCS

iPSCs provide a widely available, non-ethically disputed, and almost infinite source of pluripotent cells, providing a new paradigm in regenerative medicine stem cell maintenance and differentiation. Recently, several studies have demonstrated differentiation of reprogrammed iPSCs into fully functional RGCs. Table 2 shows a summary of experimental features of the transformation of iPSCs into RGCs. During of the RGCs differentiation process, intrinsic and extrinsic factors play key roles. Here, we reviewed factors that contribute in the differentiation of RGCs.

DIFFERENTIATION OF RETINA PROGENITOR CELLS

The vertebrate eye is formed *via* coordinated interactions between the neuroepithelium, the surface ectoderm, and the extraocular mesenchyme, which originate from the neural crest and the mesoderm[47]. Following the eye field formation, the neuroepithelium of the ventral forebrain evaginates, thus forming bilateral optic vesicles (OVs). After undergoing invagination, OVs compose distinct ocular tissues of the neural retina, the RPE, and the optic stalk[47]. During these processes, the differentiation and the fate determination of retinal cells are strictly controlled at the molecular level by cell-intrinsic transcription factors and are also influenced by cell-extrinsic signals. Previous studies show that a group of eye field transcription factors (EFTFs) are expressed in a specific region, the anterior neural plate. The EFTFs include *ET*, *Chx10*, *Rax* (also known as *Rx*), *Pax6*, *Six3*, *Six6* (also known as *Optx2*), *Lhx2*, and *Otx2*[48]. *Otx2* is required for RPE specification during eye development[49] and a group of genes encoding homeobox-containing transcription factors are thought to be at the top of the gene regulatory network during neural retina formation, such as *Chx10*, *Pax6*, *Six3*, *Six6*, and

Table 2 Summary of experimental features from iPSCs to RGCs

Cell type	Differentiation procedure	Differentiation culture medium	Genetic modification	Culture condition	RGCs markers	Time length (d)	Efficiency	Electrophysiological analysis	Transplantation	Reference
hiPSC	EBs, neurosphere, RGCs	ES medium, DAPT		Gelatin pretreated, laminin coated	Brn3a, Islet-1 Atoh7, γ-syn, Thy-1	21	27.836%±2.56%	+	N/A	[34]
hiPSC	Neural, retina-like structures, RGCs	Proneural medium, FGF2, DAPT		Xeno-free, coating-free	Brn3a, Pax6, CALRETININ	21	N/A	N/A	N/A	[20]
miPSC	EBs, RGCs	Neuronal medium, Dkk1, Noggin, DAPT	Math5 overexpression	Coating-free	Brn3b, Islet-1, Thy1, γ-syn	8	10%	N/A	N/A	[35]
miPSC	EB, neural progenitors, RGCs	NIM, NEM, RDM		PDL-coated, laminin coated	Brn3b, Math5	50	14.3%±3.439%	+	N/A	[21]
hiPSC	EBs, OV-like structures, RGCs	RDM, RMM, matrigel		Three-dimensional culture, cell-feeder	Brn3b, Atoh7, Islet1, γ-syn, Thy1	26-29	N/A	+	N/A	[36]
hiPSC	EBs, OV-like structures, RGCs	NIM, RDM		NA	Brn3, Map2	70	49.13%±2.95%	N/A	N/A	[37]
hiPSC	EBs, neurospheres, RGCs	NIM, FBS, RDM, BDNF		Coating-free	Brn3, Islet1, Map2	40	36.1%±1.7%	+	N/A	[38]
hiPSC	EBs, neurospheres, RGCs	NIM, RDM		Synthemax plates, feeder-free, xeno-free	Brn3	60	15%	N/A	N/A	[39]
hiPSC	EBs, neural aggregates, RGCs	EB medium, Dkk1, Lefty A, Noggin, RDM, DAPT	Atoh7 overexpression	Matrigel coated	Brn3b, Islet1, Atoh7	>16	23%	N/A	N/A	[40]
hiPSC	EBs, RPCs, RGCs	EB medium, differentiation medium, DAPT		Synthemax plates	Brn3b, Map2, Thy1, Atoh7	60	N/A	N/A	N/A	[41]
miPSC	EBs, OV like structures, RGCs	RDM, RMM, matrigel		Cell-feeder, PDL/ laminin coated	Brn3b, Math5, Islet1, γ-syn, Thy1	21	30%	+	N/A	[42]
hiPSC	Neural aggregates, RGCs	mTeSR1 medium, NIM, RDM		Matrigel coated	Brn3b, Tuj1, Thy1	14	10%	+	Rabbit, Rhesus monkey safe	[43]
hiPSC	Neural rosettes, RGCs	Basal medium, Shh, FGF, DAPT, CNTF		PDL/Laminin matrigel coated	Brn3b, Atoh7, Isl1	15	27.92%±13.38%	+	N/A	[44]
miPSC	EBs, RPCs, RGCs	NIM, NEM, RDM, Noggin, DKK1, Shh, FGF, DAPT		PDL/Laminin matrigel coated	Brn3b, Atoh7, Islet1, Thy1.2	15	13.3%±1.5%	+	Rat safe	[45]
miPSC	EBs, RGCs	EB medium, NIM, RDM, DAPT		Matrigel coated, PLGA scaffold	Brn3b, islet1, NF145	12	37%	N/A	N/A	[46]

hiPSC: Human iPSC; miPSC: Mouse iPSC; EB: Embryoid bodies; RGCs: Retinal ganglion cell; OV-like structures: Optic vesicle-like structures; RPCs: Retinal progenitor cells; ES: Embryonic stem; FBS: Fetal bovine serum; DAPT: N-[N-(3, 5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butylester; FGF: Fibroblast growth factor; NIM: Neural induction medium; RDM: Retinal differentiation medium; CNTF: Cilliary neurotrophic factor; RMM: Retinal maturation medium; BDNF: Brain-derived neurotrophic factor; NEM: Neural expansion medium; PDL: Poly-D-lysine; N/A: Not applicable.

Rx[50-51]. These homeobox genes are expressed in all neuroblasts at the beginning of retinogenesis and are required for the specification of RGCs, as well as other retinal cell types.

The paired-like homeobox gene *Chx10* is the earliest specific marker of neural retinal progenitor cells that is expressed in the presumptive neural retina and functions to repress the expression of the microphthalmia-associated transcription factor (*Mitf*) in the distal optic vesicle[52]. *Mitf* is a basic helix-loop-helix (HLH) transcription factor that acts as a master regulator of RPE development and is essential for the acquisition and the maintenance of RPE cells[52]. Mutations in *Chx10* cause the ocular retardation phenotype in mice[53], suggesting that *Chx10* plays critical roles in neural retinal development.

Pax6 is a paired-like homeobox gene that has maintained a high level of conservation throughout the evolution of the eye[51]. Studies have demonstrated that *Pax6* is critical during the early stages of eye development[54]. Additionally, *Pax6* can has the ability to directly activate the basic HLH transcription factor *Ath5*, the most important transcription factor in RGCs specification[55]. Loss-of-function mutations in human *Pax6* could result in could ocular syndrome aniridia[56], suggesting that *Pax6* plays a key role in eye formation.

Six3 and *Six6* are closely related members of the Six-homeodomain family. Human *Six3* mutation could result in microphthalmia and severe malformation of the brain. Mutation in *Six6* is also associated with bilateral anophthalmia[57]. These effects suggest that both *Six3* and *Six6* play important roles during retinal determination.

Rx is initially expressed throughout the anterior neural plate and later throughout the neural retina[51]. Mutations in both alleles of the mice *Rx* gene result in an inability to develop OVs, and mutation of the human gene is associated with anophthalmia and sclerocornia[58]. The *Rx* function is important during neural retina development.

Overall, these EFTFs perform roles during retina development and could be used as markers for the retina progenitor cells (RPCs) to monitor the iPSC differentiation process. In addition to these intrinsic factors, various neurotropic factors and pathways have been implicated in retina cell specification and differentiation. Elucidation of these extrinsic signaling pathways could allow researchers to more efficiently differentiate iPSCs into RGCs. These pathways include fibroblast growth factor (FGF), insulin-like growth factor (IGF), bone morphogenetic protein (BMP), nodal, and Wnt signaling pathways. These pathways all regulate the development of the neural retina, where the FGF and the IGF provide positive regulation, and the BMP, the nodal, and the Wnt signaling pathways serve as negative regulatory factors[49,52,59-60].

RETINAL GANGLION CELL DIFFERENTIATION

RGCs are the first neuronal cell type to emerge in the developing retina of vertebrates. The specification and the differentiation procedures are regulated by a group of transcription factors, including the Ath5, the Notch, and the Brn3 factors.

Ath5 (Atoh7 in humans, Math5 in mice, lakritz in zebrafish, Cath5 in chicks, and Xath5 in Xenopus) is a basic HLH transcription factor. Its expression coincides with the emerging of RGCs, where it plays a key role in the genetic regulation of RGC fate determination. Ath5 regulates the expression of the POU-domain transcription factor Brn3b and the LIM-homeodomain factor Isl1, RGC-specific differentiating transcription factors[61]. Math5 loss-of-function in mice and the lakritz mutation in zebrafish result in the nearly complete absence of RGCs, but overexpression of Cath5 in chicks and Xath5 in Xenopus promoted RGCs production[62-63], suggesting the important and irreplaceable role that Ath5 plays in RGC differentiation.

The Notch pathway is a negative regulator of RGC production that is activated by the binding of a cell-surface Notch to ligands. Notch activity in mice is down regulated just prior to the differentiation of the RGCs during normal eye development[63]. Pax6 directly activates Ath5, and Notch signaling inhibits Ath5 expression[62]. Altering Notch expression with antisense oligonucleotides in the chick retina results in an increased RGC number, where the expression of the constitutively active Notch decreased ganglion cell numbers[64]. Together, the opposing activities of Pax6 and Notch determine the accurate expression of Ath5 in a subset of cells that are proficient for RGC specification.

The class IV POU domain transcription factor Brn3b (also called Pou4f2) is downstream of Ath5 and one of the earliest markers for RGC differentiation[65]. Although remains unclear at present if Ath5 directly regulates Brn3b, Brn3b expression is significantly reduced in the Math5-null retina[66]. During mouse retinal development, the Brn3b gene is expressed in a large set of post-mitotic ganglion cell precursors and is required for both their early and terminal differentiation[67]. Brn3b-null mice exhibit programmed cell death of approximately 70% of newly formed RGCs and axon growth defects[68]. Brn3a and Brn3c (also known as Pou4f1 and Pou4f3), are two members of the class IV POU domain transcription factors. Similar to Brn3b, both Brn3a and Brn3c are expressed in differentiated RGCs during mouse retinogenesis, but two days after the onset of Brn3b expression[69]. Studies have shown that the loss function of Brn3a and Brn3c in mice does not cause retinal defects but observed some promotion of RGC axon development in the absence of Brn3b in mice and chicks. The relationship between these factors remains unclear[70]. The Brn3 factors

play important roles in RGCs differentiation, although their roles remain only partially understood.

Islet-class factor Islet-1 (also known as Isl1) is a LIM-homeodomain factor that is co-expressed with Brn3b in post-mitotic and differentiating RGCs and is also downstream of Ath5 in the gene regulatory network of RGC development[61]. Islet-1 and Brn3b exhibit essentially identical retinal expression patterns during the early stages of development and are the earliest known transcription factors that are specifically expressed in developing RGCs[71]. Retina-specific depletion of Islet-1 in mice results in the apoptosis of a majority of RGCs, as well as and in RGC axon guidance defects[61]. The specification and differentiation of RGCs occur *via* a stepwise process that involves a hierarchical gene regulatory network. These factors could be used as reliable markers for RGCs and as targets to differentiate RGCs.

γ-synuclein is highly expressed in the axons and the cytoplasm of RGCs, at the same time as Brn3a in the human retina[72]. The co-expression of Brn3a and γ-synuclein could be used as a marker of RGCs. Barnstable and Drager[73] found that Thy-1 antigen could be a ganglion cell-specific marker in rodent retina. Therefore, all of these factors could be used as RGCs markers.

CULTURE PROCEDURES AND CONDITIONS INVOLVED IN RGC DIFFERENTIATION FROM IPSCS

With detailed understanding of the molecular steps involved in RGC differentiation, investigators successfully differentiated iPSCs into RGC-like cells. Although at first the levels of expressed RGC-specific markers such as Ath5, Brn3b, Thy1, and Islet1 were not promising, significant progress has been made in generating RGC-like cells later.

CULTURE PROCEDURES

The culture and the differentiation of iPSCs nearly mimic normal eye development. The embryoid bodies (EBs) form first, followed by formation of OV-like structures or neurospheres. EBs is cellular aggregates that consist of a mixture of endodermal, mesodermal, and ectodermal cells, which are representative of the three primary germ layers in development. OV-like structures or neurospheres are proliferative cellular aggregates of neural progenitor cells that have the ability to differentiate into neurons or glia. Initially, studies demonstrated that dynamic behavior and critical development of the checkpoints in RGCs induction is an essential mean to confirm the establishment of the RGCs lineage and mimic normal eye development. Several reports indicate that these protocols require multiple steps and trained handling, which could generate undetectable variations and would not be compatible with the manufacturing process required for a therapeutic approach that might require large-scale production of cells. Reichman *et al*[20] developed a method that bypassed the EBs formation and used exogenous molecules, coating, or matrigel to simplify and shorten the procedure for RGC differentiation from hiPSCs. In this process, the neural retina-like (NR-like) structures formed first, and then developed into RGCs. Other reports demonstrated that mouse iPSCs-derived EBs express RPCs markers hierarchically and differentiate into RGCs directly[35,46]. Regardless of the step that was bypassed, these simple protocols allowed RGCs generation that were similar to native RGCs at the molecular, biochemical, and functional levels. The development of protocols that are easy-to-operate and less risky represents an important step towards effective clinical application. However, it should be noticed that those differentiation protocols that have been simplified and skip some steps do have drawbacks. First, these RGCs were largely examined the phenotype finally acquired but not the mechanisms by which they were acquired. Second, these RGCs didn't recapitulate the hierarchical regulatory mechanisms for RGC differentiation, thus predicting stability of the acquired phenotype might be affected[45].

CULTURE CONDITIONS

It is common to modify genes and/or use certain supplements and molecules to mimic *in vivo* signal pathways during RGC differentiation. N2, B27, and FGF2 are commonly added to the basal culture medium in various combinations to promote neural retina differentiation. FGF could play a role in patterning the domains of the optic vesicle and could be required for the Chx10 expression in the presumptive neural retina[74].

Nodal and BMPs are members of the TGF-β superfamily, where their pathways play key roles in antagonizing the neural anterior default differentiation program in hESCs. This demonstrates that the antagonists of the Nodal or the BMPs pathway, such as Lefty and Noggin, could be added to an RPC differentiation culture medium to ensure low levels of Nodal or BMPs and to enhance neural differentiation of iPSCs[52,60]. The Wnt signaling pathway negatively regulates neurite extension in the mouse retina, so it is not surprising that the Wnt antagonist Dickkopf (Dkk) could be added to the RPC differentiation culture medium to promote the neural differentiation of iPSCs[35,59]. The Gdf8 (a member of the TGF-β superfamily) and Hedgehog (Hh, shh in mouse) proteins are regulated by Brn3b and are extracellular signaling molecules involved in the cell non-autonomous function of RGCs, where the RGCs regulate their own production and RPC proliferation[68]. These factors help to promote neural retina differentiation from iPSCs when added to the RPC differentiation culture medium.

In addition to supplements in the culture medium, genetic modification is another strategy used to promote RGC differentiation. During RGC differentiation, Ath5 plays an irreplaceable role, so either direct or indirect genetic

modification of Ath5 could be an effective way to increase the induction of RGCs. Chen *et al*[35] found that over expression of *Math5* in mouse activated RGC-related genes in iPSCs, including *Brn3b*, *Islet-1*, and *Thy1*, indicating that Math5 overexpression stimulates the differentiation of iPSCs into RGCs. Deng *et al*[40] demonstrated that overexpression of *Atoh7* promoted RGC specification in the hiPSC-derived RPCs. Alternatively, strategies may exploit the upstream factors. For example, the Notch pathway activity is downregulated before RGC differentiation during retinogenesis, where it has been proven to inhibit Ath5 expression. Studies have explored Notch pathway inhibitors as a method to increase the expression of Ath5. The most commonly used inhibitor is N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenyl-glycine-t-butylester (DAPT)[20,35,46]. Riazifar *et al*[34] even showed that a single chemical, DAPT, could induce iPSCs to differentiate into functional RGCs.

Similar to the iPSC reprogramming procedures, the xenogeneic products for RGC growth and differentiation are essential prerequisites that enable the clinical application of iPSCs-derived RGCs. Sridhar *et al*[39] first demonstrated a xeno-free approach to differentiate hiPSCs into RGCs. Synthemax plates and nonxenogeneic Nutristem medium were used to maintain iPSCs and used mTESR1/Nutristem, Synthemax plates, and retinal differentiation medium to induce neurospheres and RGCs. Possible graft rejection risks might be caused by the use of animal products or other undefined components during routine cell culturing, so the Xeno-free approach is an attractive promising alternative. Conventional two dimensional (2D) cultures have been engineered for attachment and proliferation of iPSCs. Alternatively, three-dimensional (3D) culture system is gaining popularity, as it has the ability of self-organization, requires fewer extrinsic growth factors, and the intrinsic pattern appears to be similar to the normal eye development[36]. Existing 3D culture techniques include suspension culture, cell encapsulation in gels and cell culture in scaffolds[75-76]. Hallam *et al*[77] showed that light responsive retinal organoids derived from iPSC lines can be generated at the scale needed for pharmacology and drug screening purposes by 3D culture techniques. As RGCs lie in the innermost layer of neural retina, the techniques may explore the functional axons[43].

TRANSPLANTATION AND CONNECTION

In vivo experiment, Chen *et al*[35] successfully transplanted iPSC-derived RGCs into the mice vitreous chamber, but found almost no cells migrated into the retina. It was thought that this could be due to the normal retinal environment that acts as a barrier for graft integration and the formation of functional synapses. However, Parameswaran *et al*[45] indicated that following transplantation of the iPSC-derived RGCs in the rat model of ocular hypertension, cells were incorporated into the host RGC layer and expressed RGC-specific markers without producing tumors. Hertz *et al*[78] demonstrated that RPC-derived RGCs exhibit a similar capacity for integration as developing primary RGCs, where they appear to form a lower number of presynaptic punctae in both normal and optic nerve axotomy rats models. Although the effects of cell transplantation therapy are not clearly understood, cell transplantation could offer a feasible strategy for neuroprotective and cell-replacement therapy.

The function of the RGCs as projection neurons of the retina is dependent on their ability to form contacts with central targets. An important challenge for iPSC-derived RGCs transplantation is for these cells to integrate in the ganglion cell layer (GCL) and to also develop functional axons that connect to the optic nerve and possibly form connections with the brain. iPSC-derived RGCs with functional axons have been successfully established[34,36,42]. Teotia *et al*[44] showed that iPSC-derived RGCs *in vitro* expressed a series of guidance molecules that could guide the axons in the retina, at the optic chiasm, and even in the central targets. Tissue engineering is now used for treatment of glaucoma and other retinal diseases by using a medical polymer biomaterial as a substrate for cell seeding and delivery. For example, the use of electrospun[79], Netrin-1[80], or 3D-printed scaffolds[81] have been used to control RGCs neurite growth. Li *et al*[43] transplanted of an engineered iPSC-derived RGCs scaffold biomaterial of biodegradable poly (lactic-co-glycolic acid) (PLGA) into the retinal surfaces of rabbits and rhesus monkeys, which resulted in dendritic arbors, prominent axons, neurite networks, and altered electrophysiological properties. The cell-scaffold could be relevant for potential clinical applications within tissue engineering therapy and retinal surface delivery, however, the vitrectomy needed to guarantee effective adhesive remains a challenge.

PERSPECTIVES AND CONCLUSIONS

Additional optimization of the process to generate iPSC-derived RGCs is required for clinical applications. First, the primitive hiPSCs used to differentiate into RGCs should be patient-specific and should be reprogrammed under feeder-free, xeno-free, and integration-free conditions to avoid the risk of graft rejection and genotoxicity. Second, the medium devoid of xenogeneic components should be used for RGCs differentiation when specifying RPCs and RGCs. Third, high-efficiency, simple and reproducible protocols should be established, where predictable variations should be minimized during each step. Fourth, the hiPSC-derived RGCs should be easily translatable to an *in vivo* environment, and the brain connection should be established.

In conclusion, some significant challenges still exist, such as the risk of teratocarcinoma formation. A lack of robust and highly reproducible differentiation protocols and accurate axon

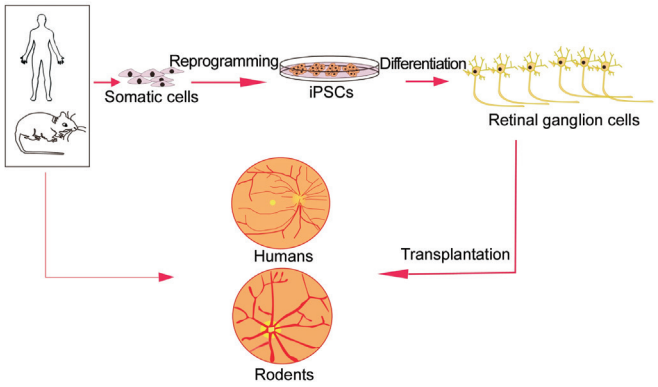

Figure 1 Flow diagram of personalized treatment for glaucoma and other optic nerve diseases.

guidance still exists. However, iPSC-derived RGCs remain a potentially life-changing tool for the study of RGCs biology and the eventual personalized treatment for glaucoma and other optic nerve diseases (Figure 1).

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