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

<table>
<thead>
<tr>
<th>Cell source</th>
<th>Culture condition</th>
<th>Reprogramming factors</th>
<th>Delivery method</th>
<th>Time length (d)</th>
<th>Efficiency (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse fibroblasts</td>
<td>Cell feeder</td>
<td>Oct3/4, Sox2, C-myc, Klf4</td>
<td>Retrovirus</td>
<td>N/A</td>
<td>0.02</td>
<td>[9]</td>
</tr>
<tr>
<td>Human fibroblasts</td>
<td>Cell feeder</td>
<td>Oct4, Sox2, Nanog, Lin28</td>
<td>Lentivirus</td>
<td>15</td>
<td>0.022</td>
<td>[22]</td>
</tr>
<tr>
<td>Human fibroblasts</td>
<td>Cell feeder</td>
<td>Oct3/4, Sox2, Klf4, Sall4</td>
<td>Retrovirus</td>
<td>25</td>
<td>0.014</td>
<td>[23]</td>
</tr>
<tr>
<td>Human blood mononuclear cells</td>
<td>Cell feeder</td>
<td>Oct4, Sox2, Klf4, C-myc, Lin28</td>
<td>Plasmid</td>
<td>14</td>
<td>0.05</td>
<td>[11]</td>
</tr>
<tr>
<td>Human fibroblasts</td>
<td>Cell feeder</td>
<td>Oct3/4, Sox2, Klf4, Gisil1</td>
<td>Retrovirus</td>
<td>22</td>
<td>0.17</td>
<td>[24]</td>
</tr>
<tr>
<td>Human fibroblasts</td>
<td>Cell feeder</td>
<td>Oct3/4, Sox2, Klf4, L-myc, Lin28, P53</td>
<td>Plasmid, shRNA</td>
<td>14</td>
<td>0.03</td>
<td>[25]</td>
</tr>
<tr>
<td>Human keratinocytes</td>
<td>Cell feeder</td>
<td>Oct4, Sox2, Klf4, C-myc, Rem2</td>
<td>Retrovirus</td>
<td>12</td>
<td>0.2</td>
<td>[26]</td>
</tr>
<tr>
<td>Human fibroblasts</td>
<td>Cell feeder</td>
<td>Oct4, Sox2, Myc, Klf4, Sv40 large T antigen</td>
<td>Lentivirus</td>
<td>14</td>
<td>0.46</td>
<td>[27]</td>
</tr>
<tr>
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<td>Cell feeder</td>
<td>Oct3/4, Sox2, C-myc, Klf4</td>
<td>Adenovirus</td>
<td>25</td>
<td>0.0002</td>
<td>[28]</td>
</tr>
<tr>
<td>Mouse and human fibroblasts</td>
<td>Cell feeder</td>
<td>Oct3/4, Sox2, C-myc, Klf4</td>
<td>2A peptide</td>
<td>9</td>
<td>2.5</td>
<td>[13]</td>
</tr>
<tr>
<td>Human fibroblasts</td>
<td>Cell feeder</td>
<td>Oct3/4, Sox2, C-myc, Klf4</td>
<td>PiggyBac transposon</td>
<td>14</td>
<td>0.01</td>
<td>[13]</td>
</tr>
<tr>
<td>Human fibroblasts</td>
<td>Cell feeder</td>
<td>Oct3/4, Sox2, C-myc, Klf4</td>
<td>PiggyBac transposon</td>
<td>10-14</td>
<td>0.03</td>
<td>[29]</td>
</tr>
<tr>
<td>Human adipose stromal cells</td>
<td>Cell feeder</td>
<td>Oct4, Sox2, Nanog, Lin28</td>
<td>Minicircle DNA</td>
<td>18</td>
<td>0.0075</td>
<td>[30]</td>
</tr>
<tr>
<td>Human fibroblasts</td>
<td>Xeno-free, feeder-free</td>
<td>Oct3/4, Sox2, Klf4, Lin28, TP53, A-83-01, sodium butyrate, CHIR99021</td>
<td>Plasmids, shRNA, small molecules</td>
<td>16</td>
<td>0.1</td>
<td>[10]</td>
</tr>
<tr>
<td>Mouse fibroblasts</td>
<td>Cell feeder</td>
<td>Mir-200c, Mir-302s, Mir-369s</td>
<td>miRNA</td>
<td>15</td>
<td>0.1</td>
<td>[12]</td>
</tr>
<tr>
<td>Human fibroblasts</td>
<td>Cell feeder</td>
<td>Mir-200c, Mir-302s, Mir-369s</td>
<td>miRNA</td>
<td>20</td>
<td>0.002</td>
<td>[12]</td>
</tr>
<tr>
<td>Human fibroblasts</td>
<td>Cell feeder</td>
<td>Oct4, Sox2, VPA</td>
<td>Retrovirus, small molecules</td>
<td>N/A</td>
<td>0.01</td>
<td>[31]</td>
</tr>
<tr>
<td>Human fibroblasts</td>
<td>Cell feeder</td>
<td>Oct4, Sox2, Klf4, VPA</td>
<td>Retrovirus, small molecules</td>
<td>N/A</td>
<td>1</td>
<td>[31]</td>
</tr>
<tr>
<td>Human bone mesenchymal stromal cells</td>
<td>Feeder-free</td>
<td>Oct4, Sox2, Nanog, Lin28, Sv40t, Klf4, C-myc, A-83-01, PD325901, CHIR99021, HA-100</td>
<td>Plasmids, small molecules</td>
<td>N/A</td>
<td>N/A</td>
<td>[18]</td>
</tr>
<tr>
<td>Mouse fibroblasts</td>
<td>Cell feeder</td>
<td>Oct4, tranylcypromine, VPA, CHIR99021, 616452</td>
<td>Lentivirus, small molecules</td>
<td>18d</td>
<td>0.03</td>
<td>[32]</td>
</tr>
<tr>
<td>Human fibroblasts</td>
<td>Xeno-free, feeder-free</td>
<td>Oct3/4, Sox2, C-myc, Klf4</td>
<td>Lentivirus</td>
<td>7-14d</td>
<td>0.3</td>
<td>[33]</td>
</tr>
</tbody>
</table>

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 the RGCs differentiation process, intrinsic and extrinsic factors play key roles. Here, we reviewed factors that contribute to 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>
<thead>
<tr>
<th>Cell type</th>
<th>Differentiation procedure</th>
<th>Differentiation culture medium</th>
<th>Genetic modification</th>
<th>Culture condition</th>
<th>RGCs markers</th>
<th>Time length (d)</th>
<th>Efficiency</th>
<th>Electrophysiological analysis</th>
<th>Transplantation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>hiPSC: EBs, neurosphere, RGCs</td>
<td>ES medium, DAPT</td>
<td>Gelatin pretreated, laminin coated</td>
<td>Brn3a, Islet-1, Atoh7, γ-syn, Thy-1</td>
<td>21</td>
<td>27.836±2.58%</td>
<td>+</td>
<td>N/A</td>
<td>[34]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hiPSC: Neural, retina-like structures, RGCs</td>
<td>Proneural medium, FGF2, DAPT</td>
<td>Xeno-free, coating-free</td>
<td>Brn3a, Pax6, CALRETININ</td>
<td>21</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miPSC: EBs, RGCs</td>
<td>Neuronal medium, Dkk1, Noggin, DAPT</td>
<td>Matth5 overexpression</td>
<td>Coating-free</td>
<td>Brn3b, Islet-1, Thy1, γ-syn</td>
<td>8</td>
<td>10%</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>miPSC: EB, neural progenitors, RGCs</td>
<td>NIM, NEM, RDM</td>
<td>PDL-coated, laminin coated</td>
<td>Brn3b, Math5</td>
<td>50</td>
<td>14.3±3.43%</td>
<td>+</td>
<td>N/A</td>
<td>[21]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hiPSC: EBs, OV-like structures, RGCs</td>
<td>RDM, RMM, matrigel</td>
<td>Three-dimensional culture, cell-feeder</td>
<td>Brn3b, Atoh7, Islet1, γ-syn, Thy1</td>
<td>26-29</td>
<td>N/A</td>
<td>+</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hiPSC: EBs, OV-like structures, RGCs</td>
<td>NIM, RDM</td>
<td>NA</td>
<td>Brn3, Map2</td>
<td>70</td>
<td>49.13±2.95%</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hiPSC: EBs, neurospheres, RGCs</td>
<td>NIM, FBS, RDM, BDNF</td>
<td>Coating-free</td>
<td>Brn3, Islet1, Map2</td>
<td>40</td>
<td>36.1±1.7%</td>
<td>+</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hiPSC: EBs, neurospheres, RGCs</td>
<td>NIM, RDM</td>
<td>Synthemax plates, feeder-free, xeno-free</td>
<td>Brm3</td>
<td>60</td>
<td>15%</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hiPSC: EBs, neural aggregates, RGCs</td>
<td>EB medium, Dkk1, Lefty A, Noggin, RDM, DAPT</td>
<td>Atoh7 overexpression</td>
<td>Matrigel coated</td>
<td>Brn3b, Islet1, Atoh7</td>
<td>&gt;16</td>
<td>23%</td>
<td>N/A</td>
<td>N/A</td>
<td>[40]</td>
<td></td>
</tr>
<tr>
<td>hiPSC: EBs, RPCs, RGCs</td>
<td>EB medium, differentiation medium, DAPT</td>
<td>Synthemax plates</td>
<td>Brn3b, Map2, Thy1, Atoh7</td>
<td>60</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miPSC: EBs, OV-like structures, RGCs</td>
<td>RDM, RMM, matrigel</td>
<td>Cell-feeder, PDL/ laminin coated</td>
<td>Brn3b, Math5, Islet1, γ-syn, Thy1</td>
<td>21</td>
<td>30%</td>
<td>+</td>
<td>N/A</td>
<td>N/A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hiPSC: Neural aggregates, RGCs</td>
<td>mTeSR1 medium, NIM, RDM</td>
<td>Matrigel coated</td>
<td>Brn3b, Tuji, Thy1</td>
<td>14</td>
<td>10%</td>
<td>+</td>
<td>Rabbit, Rhesus monkey safe</td>
<td>[43]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hiPSC: Neural rosettes, RGCs</td>
<td>Basal medium, Shh, FGF, DAPT, CNTF</td>
<td>PDL/Laminin matrigel coated</td>
<td>Brn3b, Atoh7, Isl1</td>
<td>15</td>
<td>27.92±13.38%</td>
<td>+</td>
<td>N/A</td>
<td>[44]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miPSC: EBs, RPCs, RGCs</td>
<td>NIM, NEM, RDM, Noggin, DKK1, Shh, FGF, DAPT</td>
<td>PDL/Laminin matrigel coated</td>
<td>Brn3b, Atoh7, Islet1, Thy1</td>
<td>15</td>
<td>13.3±1.5%</td>
<td>+</td>
<td>Rat safe</td>
<td>[45]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>miPSC: EBs, RGCs</td>
<td>EB medium, NIM, RDM, DAPT</td>
<td>Matrigel coated, PLGA scaffold</td>
<td>Brn3b, Islet1, NF145</td>
<td>12</td>
<td>37%</td>
<td>N/A</td>
<td>N/A</td>
<td>[46]</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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: Ciliary neurotrophic factor; RMM: Retinal maturation medium; BDNF: Brain-derived neurotrophic factor; NEM: Neural expansion medium; PDL: Poly-D-lysine; N/A: Not applicable.
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\(^\text{[52]}\). 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\(^\text{[52]}\). Mutations in Chx10 cause the ocular retardation phenotype in mice\(^\text{[52]}\), suggesting that Chx10 plays critical roles in neural retinal development.

\(\text{Pax6}\) is a paired-like homeobox gene that has maintained a high level of conservation throughout the evolution of the eye\(^\text{[51]}\). Studies have demonstrated that \(\text{Pax6}\) is critical during the early stages of eye development\(^\text{[44]}\). Additionally, \(\text{Pax6}\) can has the ability to directly activate the basic HLH transcription factor \(\text{Ath5}\), the most important transcription factor in RGCs specification\(^\text{[55]}\). Loss-of-function mutations in human \(\text{Pax6}\) could result in ocular aniridia\(^\text{[56]}\), suggesting that \(\text{Pax6}\) plays a key role in eye formation.

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

\(\text{Rx}\) is initially expressed throughout the anterior neural plate and later throughout the neural retina\(^\text{[51]}\). Mutations in both alleles of the mouse \(\text{Rx}\) gene result in an inability to develop OVs, and mutation of the human gene is associated with anophthalmia and microphthalmia\(^\text{[58]}\). The \(\text{Rx}\) 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\(^\text{[49,52,59-60]}\).

### 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 \(\text{Ath5}\), the Notch, and the \(\text{Bm3}\) factors.

\(\text{Ath5}\) (Atoh7 in humans, \(\text{Math5}\) in mice, \(\text{lakritz}\) in zebrafish, \(\text{Cath5}\) in chicks, and \(\text{Xath5}\) 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. \(\text{Ath5}\) regulates the expression of the POU-domain transcription factor \(\text{Brn3b}\) and the LIM-homeodomain factor \(\text{Isil}\), RGC-specific differentiating transcription factors\(^\text{[61]}\). \(\text{Math5}\) loss-of-function in mice and the \(\text{lakritz}\) mutation in zebrafish result in the nearly complete absence of RGCs, but overexpression of \(\text{Cath5}\) in chicks and \(\text{Xath5}\) in Xenopus promoted RGCs production\(^\text{[62-63]}\), suggesting the important and irreplaceable role that \(\text{Ath5}\) 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\(^\text{[63]}\). \(\text{Pax6}\) directly activates \(\text{Ath5}\), and Notch signaling inhibits \(\text{Ath5}\) expression\(^\text{[62]}\). 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\(^\text{[64]}\). Together, the opposing activities of \(\text{Pax6}\) and Notch determine the accurate expression of \(\text{Ath5}\) in a subset of cells that are proficient for RGC specification.

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

**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. 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. 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. 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. 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-butylerester (DAPT). 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 iPSC-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[41].

**TRANSLATION 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[38] 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 anatomy 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
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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**REFERENCES**


33 Lu HF, Chai C, Lim TC, Leong MF, Lim JK, Gao S, Lim KL, Wan AC. A defined xeno-free and feeder-free culture system for the derivation, expansion and direct differentiation of transgene-free patient-specific induced pluripotent stem cells. *Biomaterials* 2014;35(9):2816-2826.


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