Methods of Retinal Ganglion Cell Differentiation From Pluripotent Stem Cells

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Introduction

Glaucoma, the worldwide leading cause of irreversible blindness, is characterized by progressive degeneration of the optic nerve and loss of retinal ganglion cells. Research into glaucoma pathogenesis has been hampered by difficulties in isolating and culturing retinal ganglion cells in vitro. However, recent improvements in laboratory techniques have enabled the generation of a variety of mature cell types from pluripotent stem cells, including retinal ganglion cells. Indeed, stem cell-based approaches have the potential to revolutionize the field by providing an unlimited source of cells for replacement therapies and by enabling development of in vitro disease models for drug screening and research. Consequently, research aimed at directing pluripotent stem cells to differentiate into retinal ganglion cells has expanded dramatically during the past decade, resulting in significant advances in technique and efficiency. In this paper, we review the methodology for retinal ganglion cell differentiation from pluripotent stem cells of both mouse and human origin and summarize how these techniques have opened up new avenues for modelling glaucoma. Generation of stem cell–derived retinal ganglion cells will have significant translational values, providing an in vitro platform to study the mechanisms responsible for pathogenesis and for drug screening to improve treatment options, as well as for the development of cell therapies for optic neuropathies such as glaucoma.

Glaucoma is the leading cause of irreversible blindness worldwide.1 It is predicted that by 2020, there will be approximately 80 million people with the disease.1 Glaucoma is a broad term for a group of ocular diseases characterized by loss of retinal ganglion cells (RGCs) and their axons that comprise the optic nerve.2 Clinical characteristics of the primary open-angle glaucoma include progressive excavation of the optic nerve head with corresponding visual loss.2–4 The cause and precise pathophysiology of primary open-angle glaucoma are poorly understood, though a number of risk factors have been implicated.5,6 Several theories have been developed to explain glaucoma-based RGC loss, including increased intraocular pressure (IOP), vascular dysregulation, genetics, neurotrophic factor deficit, mitochondrial malfunction, inflammation, as well as increased oxidative stress.7,8 Due to the irreversible nature of RGC axonal loss, there is currently a limited arsenal for treatment. Treatment typically includes reduction of increased IOP via topical therapy, laser treatment, or surgery.5,9

A major barrier to studying and treating eye diseases such as glaucoma is the difficulty of sampling RGCs from patients premortem. Thus, generation of an in vitro RGC system is crucial to advance research in optic neuropathies. In the retina, Müller cells represent the endogenous stem/progenitor cells and possess the ability to differentiate into RGCs.10,11 However, currently, Müller cells cannot be maintained in vitro for a prolonged period, and are thus of limited use for the generation of RGCs in vitro. Also, Müller cells need to be extracted from patients, which are of limited supply. Pluripotent stem cells (PSCs) have the ability to differentiate into all cell types of the human body and can be maintained in vitro
Thus, PSCs represent an unlimited source for generation of specific cell types including RGCs. Aside from their potential use for transplantation, stem cell–derived RGCs may also be used for disease modeling and subsequently for drug screening. A pioneering discovery came in 2006 whereby adult somatic cells were successfully reprogrammed into induced pluripotent stem cells (iPSCs) by expressing four factors (OCT4, SOX2, KLF4, and MYC) in mouse, and later human, fibroblasts. While functionally similar to embryonic stem cells (ESCs), iPSCs have broadened the scope for disease- or injury-based cellular therapies because patient-derived iPSCs should be genetically and immunologically matched to the patient. Several studies have demonstrated successful generation of mature retinal cell types, including RGC-like cells, from differentiated rodent and human PSCs (summarized in Tables 1, 2) that may be used to understand the survival and maintenance of retinal cells in vitro and provide a model system to study glaucoma pathogenesis.

The cell bodies of RGCs are located in the inner retina, with their axons projecting through the optic nerve back to the lateral geniculate nucleus. Structurally, RGCs vary in size and morphology, with at least 20 subtypes being described in the mouse. Following a high degree of preretinal processing, RGCs transmit visual information (such as color, movement, direction, and contrast) along the optic nerve to the midbrain where they synapse. Within the optic disc, RGC axons typically remain unmyelinated until they pass through the lamina cribrosa, a small window of collagen. Beyond the lamina cribrosa, RGCs converge to form the optic nerve fibers where they become myelinated by oligodendrocytes. Also present within the optic nerve, astrocytes maintain the blood–retinal barrier and activate the inflammatory system via release of cytokines. Together, oligoden-
RGCs are mitochondria-rich, anterior to the lamina cribrosa yet the number of mitochondria declines once the axons become myelinated, indicating an inverse relationship between mitochondria and myelin in these neurons. Therefore, the soma and unmyelinated regions of RGCs have a high metabolic rate and energy consumption while the myelinated regions require less energy. RGCs differentiated from cellular sources, such as PSCs, therefore provide an invaluable tool for studying RGC development and survival in vitro that may expose underlying disease-related mechanisms for RGC senescence and apoptosis. As a prerequisite for these disease studies, researchers must first be able to generate sufficient numbers of functional RGCs from PSCs. Here, we review current techniques for retinal neural differentiation from PSC cultures, both human and nonhuman derived, and we discuss future directions for this research.

### Markers to Identify Retinal Differentiation of Stem Cells

During retinal differentiation from PSC cultures, molecular and protein markers are needed to identify retinal progenitor cells (RPCs) and mature retinal cells. Here, we review markers that can be used for identification of RPCs and RGCs.

### Eye Field Markers

During development of the embryo, eye field transcription factors (EFTFs) specify development of the eye. The eye field is denoted by overlapping expression of the EFTFs within a specific region of the anterior neural plate. These EFTFs are useful to indicate early retinal differentiation from PSCs. It is important to note that although these EFTFs are reliable markers to identify RPCs, they are not necessary useful for identification of RGCs as their...
expression may change during differentiation from RPCs to RGCs. EFTFs expressed in vertebrates include: paired box 6 (PAX6), retinal and anterior neural fold homeobox (RAX, also known as RX), SIX homeobox 3 (SIX3), SIX homeobox 6 (SIX6), Et (also known as T-box transcription factor [TBX3]), and tailless/TLX (see Refs. 23, 24 for review).

PAX6 is initially expressed within the optic vesicle and its expression persists in the lens placode ectoderm and the inner retinal layers including RGCs.25,26 Haploinsufficiency mutations within the PAX6 gene result in the development of aniridia27 while homozygous mutations in PAX6 result in anophthalmia28 suggesting PAX6 plays a role in lens and eye formation.

RAX is another marker for RPCs during development and defects in RAX result in severe effects on eye formation. Mutations in both alleles of human RAX are associated with anophthalmia and sclerocoria29 and Rax-knockout mice fail to develop eyes.30 Constitutive overexpression of rax in Xenopus embryos indicates that rax may be involved in the generation and/or proliferation of RPCs.30 Taken together, the loss-of-function and gain-of-function studies indicate a clear role for Rax in development.

SIX3 and SIX6 are initially expressed in the developing optic vesicle32 and later in the retinal ganglion and inner neural layers of the immature retina.33 Both SIX3 and SIX6 are required for eye development as mutations in human SIX3 results in microphthalmia and severe malformation of the

Table 2. Published Retinal Ganglion Cell Differentiation Protocols Used on Human Pluripotent Stem Cells

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Differentiation Culture Media</th>
<th>Isolation Method</th>
<th>mRNA Markers</th>
<th>Protein Markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>hESC + hiPSC</td>
<td>20% KSR, FGF2, N2 supplement, B27 supplement</td>
<td>NA</td>
<td>OV expressed: BRN3. No retinal specific genes expressed in EFB.</td>
<td>OV: BRN3B, TUBB3, VSBX2. EFB: PAX6, TUBB3, GABA.</td>
</tr>
<tr>
<td>hESC</td>
<td>10% KSR, B27 supplement, noggin, DKK1, IGF1, N2 supplement, FGF2.</td>
<td>NA</td>
<td>LHX2, PAX6, RAX, SIX3, ATOH7.</td>
<td>PAX6, VSBX2, SOX2 (not shown), ELAV3/4, NEFM, TUBB3.</td>
</tr>
<tr>
<td>hESC</td>
<td>NBM, B27 supplement, N2 supplement, noggin, FGF2, DKK1, IGF1, FGF9.</td>
<td>NA</td>
<td>RAX, SIX6, PAX6, VSX2, POU4F2, MITF, ATOH7,</td>
<td>Non-transplanted differentiated hESC: NES, TUBB3. Grafts: TUBB3, SYT.</td>
</tr>
<tr>
<td>hiPSC</td>
<td>10% KSR, B27 supplement, N2 supplement, noggin, DKK1, IGF1, FGF2, FGF2, DAPT.</td>
<td>NA</td>
<td>PAX6, VSX2.</td>
<td>SOX1, SOX2, PAX6, OTX2, LHX2, SIX6, VSX2, TUBB3, BRN3B.</td>
</tr>
<tr>
<td>hiPSC</td>
<td>1) 20% KSR, FGF2. 2) Feeder-free: mTESR1 medium. 3) Xeno-free: Nutristem medium, B27 supplement.</td>
<td>NA</td>
<td>Xeno-free: PAX6, SOX1, SOX2, OTX2. Feeder-free: PAX6, SOX1, SOX2, OTX2, RAX, SIX3. MEF: PAX6, OTX2, RAX, LHX2.</td>
<td>SOX1, SOX2, PAX6, OTX2, LHX2, SIX6, VSX2, TUBB3, BRN3B.</td>
</tr>
<tr>
<td>hESC + hiPSC</td>
<td>20% KSR, FGF2, 10% FBS, B27 supplement, DAPT.</td>
<td>NA</td>
<td>ATOH7, BRN3B</td>
<td>hESC: PAX6, RX, TUBB3, ISL1, BRN3B, THY1, γ-synuclein. hiPSC: TUBB3, ISL1, BRN3B, THY1, γ-synuclein.</td>
</tr>
</tbody>
</table>

Cell type refers to the pluripotent stem cell source.
hiPSC, human iPSC; hESC, human ESC; miPSC, mouse iPSC.
brain, while haploinsufficiency within \textit{SIX6} is associated with bilateral anophthalmia.

Other members of EFTFs include ET/TBX3, a T-box transcription factor that functions as an important regulator of eye field specification in \textit{Xenopus}. Developmental study in \textit{Xenopus} demonstrated that ET/TBX3 is expressed in the developing optic vesicles, ventral forebrain, and otic vesicles. TLL is first expressed in the prechordal region of the neural plate and has partial overlap expression with the eye field region. In \textit{Xenopus}, TLL can induce expression of other EFTFs such as PAX6 and SIX3. In summary, these EFTFs may be used as biomarkers for identification of the course of retinal differentiation from PSCs.

### RGC Markers

Many mature retinal cell types, including first-born RGCs, are derived from multipotent RPCs. Identification of RGCs requires confirmed expression of a combination of markers because while all are expressed in RGCs, some are also expressed in other cell types outside of the eye. Published RGC markers include $\beta$III TUBULIN (TUBB3), POU class 4 homeobox 1 (POU4F1, also known as BRN3A), POU class 4 homeobox 2 (POU4F2, also known as BRN3B), ISLET1, atonal homolog 7 (ATOH7, also known as Math5 in mouse), HUC, HUD, thy-1 cell surface antigen (THY1), medium polypeptide neurofilament (NEFM), ribosome production factor 1 homolog (RPF1), and opsin 4 (OPN4, also known as melanopsin). $\beta$III TUBULIN, ELAV 3/4, and NEFM are expressed in other neuronal cells, and therefore not exclusive to RGCs. OPN4 is expressed by a small subset of intrinsically photosensitive RGCs. Atoh7 is expressed at the onset of RGC differentiation. Downstream of Atoh7, and mediating RGC differentiation, are the transcription factors...
Brn3b and ISL LIM homeobox 1 (Isl1, also known as ISLET1). Brn3b is expressed in most RGC subtypes, and thus serves as a common RGC marker. Functionally, Brn3b is required for specification of RGC fate from RPCs. The functions of Brn3a, Brn3b, and Brn3c transcription factors are conserved in mouse and human neural development; however, Brn3a and Brn3c are only expressed after the onset of RGC differentiation. Isl1 is another transcription factor expressed in several tissues during development, including the retina. In mice, Isl1 is expressed after the initial wave of RGC generation to modulate differentiation and survival. Thy1 is a plasma membrane glycoprotein that is expressed on most RGCs within the retina and also on a range of other cells throughout the body including amacrine and Müller cells in the retina. Nevertheless, Thy1 is frequently used as a surface marker to isolate primary RGCs from eye samples. While the exact function of Thy1 within the retina and in RGCs is unclear, it has been implicated in retinal development and neurite outgrowth.

Importantly, expression of many of these RGC markers discussed is not specific to RGCs only. For instance, members of Brn3 are also expressed in auditory neurons and somatosensory neurons. Therefore, while expression of a single marker may be sufficient to identify the RGC layer within the retina, this may not be the case for identifying RGCs from PSC differentiation. Thus, it is important to use multiple markers to identify RGCs derived from pluripotent source. Future research into identification of novel markers specific to RGCs will also help address this issue. In addition to using biomarkers, other cell biology assays can be used to identify PSC-derived RGCs. For instance, glutamate-induced calcium response is a good assay to characterize the glutamatergic nature of the derived RGCs.

## Differentiation of RGCs from PSCs

### Directed Differentiation Through Culture Medium Conditions

To date, the generation of RGC populations from PSCs, including ESCs and iPSCs, has achieved some success. Many protocols first initiate differentiation of PSCs through suspension culture by forming embryoid bodies (EB) or neurospheres. EBs are cellular aggregates that consist of a mixture of endodermal, mesodermal, and ectodermal cells, representative of the three primary germ layers in development. Neurospheres are proliferative spheres of neural stem/progenitor cells (NS/PCs) that can differentiate into neurons or glia. A common method for generating RGCs in vitro is to direct PSCs to differentiate toward a retinal cell lineage using specific growth factors or molecules to mimic signals during retinogenesis. During formation of the optic cup, RGCs are the first cell type to develop in the neural retina. Retinogenesis is a complex, highly regulated process involving several signaling pathways including fibroblast growth factor (FGF) signaling, insulin-like growth factor (IGF) signaling, epidermal growth factor (EGF) signaling, bone morphogenetic protein (BMP) signaling, nodal signaling, Wnt signaling, and notch signaling pathways. Using a combination of small molecules and/or growth factors to modulate these signaling pathways, PSCs can be directed to differentiate into RPCs and subsequently RGCs. Common factors added to media to induce differentiation include: FGF2, IGF1, EGF, the BMP antagonist noggin, the nodal antagonist left-right determination factor 2 (LEFTY2), and the Wnt-signaling pathway inhibitor dickkopf 1 (DKK1; Tables 1, 2). In addition, N266–70 and B2766,69 supplements are commonly added to the basal culture media to enhance neural retina differentiation. Until recently, the majority of experimental procedures typically used mouse ESCs or iPSCs. FGF2 is often used to induce RGC differentiation in mouse ESCs/iPSCs. FGF2 promotes RGC differentiation of neural progenitors derived from mouse ESCs when cultured with sonic hedgehog (Shh), as demonstrated by expression of the RGC markers Atoh7, Brn3b, Rpf1, Thy1, and Isl1. When NS/PCs cultured in the presence of FGF2 are injected as a single cell suspension intravitreally into rat eyes, they integrate into the ganglion cell layer of the host retina, express Atoh7, and downregulate expression of the NS/PC marker nestin, which suggests acquisition of a RGC-like phenotype. Other small molecules/growth factors are also reported for RGC differentiation in mouse ESCs/iPSCs, including noggin, Dkk1 and left-right determination factor 2 (LEFTY2; Table 1). In mouse iPSCs, noggin is used to maintain and expand RPCs. Dkk1 and the nodal antagonist Lefty2 promote neural and telencephalic differentiation of mouse ESCs, suggesting that Wnt and nodal pathways negatively regulate anterior central nervous system (CNS) development. Treatment of mouse ESC-derived EBs with Lefty2 and Dkk1 further promotes the expression of RPC markers Rax and Pax6. Mouse iPSC-derived EBs
treated with Lefty2 and Dkk1 adopt a neuronal-like morphology and express Pax6; however, these cells fail to differentiate into RGC-like cells and lack expression of Brn3b, IsIl, and Thy1.2. Another molecule, N-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenylglycine-1,1-dimethylethyl ester (DAPT) is added as a notch-pathway inhibitor (∇-secretase inhibitor). Application of DAPT pushes Math5 overexpressing mouse iPSCs toward an RGC fate displaying RGC morphology and expression of the protein markers Math5, Brn3, and Thy1.2.

In recent years, studies of RGC differentiation using human ESCs and iPSCs have gained momentum. Many of these RGC differentiation protocols use basal culture media consist of Dulbecco’s Modified Eagle Medium (DMEM), Glasgow’s MEM (GMEM), or neurobasal media, supplemented with 10% to 20% knock-out serum replacement (KSR), N2, and B27. Generally, a cocktail of growth factors/small molecules are used to induce RGC differentiation in human PSCs. These include FGF2, IGF1, DKK1, and DAPT, (Table 2). Several lines of evidence exist for their addition into differentiation culture. In human ESCs, FGF2 modulates expression of the Pax6 via the ERK pathway. IGF1 also appears to be a positive regulator of RPC induction, as expression of RPC genes were reduced in its absence. Furthermore, inhibition of Wnt signaling by the small molecule CK1-7 in human ESC-derived EBs is reported to promote RPC differentiation, as indicated by the expression of RAX, Pax6, microphthalmia-associated transcription factor (MITF) and visual system homeobox 2 (VSX2). Other studies have showed that LEFTY2 and DKK1 promote differentiation of human iPSC-derived EBs into RPCs as evidenced by expression RX, Pax6, and MITF.

Importantly, an elegant study by Lamba et al. reported RGC differentiation from human ESCs using a cocktail of growth factors/antagonists. Human ESC-derived EBs treated with a combination of noggin, DKK1, and IGF1 induces an anterior neural cell fate. Treatment with these molecules increases expression of RPC markers Pax6, RAX, LIM homeobox 2 (LHX2), and SIX3, followed by expression of RGC markers ELAV3/4, NEFM, and TUBB3. In addition, the authors demonstrated functional glutamate receptor activities in the derived RGCs, which is a characteristic of mature inner retinal neurons. Subsequently, Tucker et al. showed that human iPSC-derived EBs treated with DAPT, FGF2, FGF1, DKK1, noggin, and IGF1 successfully differentiated into RGC-like cells marked by expression of NF200 and BRN3B. In a recent study, Riazifar et al. demonstrated a stepwise chemically-induced protocol for RGC differentiation from human ESCs and iPSCs, yielding approximately 30% of RGC differentiation efficiency. These stem cell–derived RGCs express markers including BRN3A, BRN3B, ATOH7/Math5, ISL1, THY1, and γ-synuclein, and an electrophysiology profile of functional and mature RGCs.

In summary, the human PSC differentiation literature indicates that a combination of supplements is required for successful generation of RGC-like cells and other retinal cell types including RPCs. Despite multiple reports of RGC differentiation from human ESCs and iPSCs as discussed in this review, the existing protocols often have low efficiency of RGC differentiation and yield heterogeneous populations of retinal cells. Future research to refine existing protocols to efficiently generate RGCs from human PSCs with high purity will enable disease modelling for optic neuropathies and prove helpful in realizing the clinical potential for PSCs.

Directed Differentiation Through Genetic Modifications

Another method to direct RGC differentiation of PSCs involves overexpression of transcription factors vital to RGC development. For instance, Pax6 overexpression has been performed in mouse ESC and iPSC lines to push undifferentiated cells into RPCs. Pax6 overexpression in mouse ESCs results in a purified culture of neural cells that express the NS/PC markers nestin and musashi RNA-binding protein 1 (Msi1). After 4 days in differentiation culture media, NS/PCs express the neuronal markers Tubb3, Nefm, and Nestin. Ten days later, a large proportion of differentiated cells are positive for Tubb3 and Brn3, while a small proportion are positive for the RGC markers Thy1, IsIl, and Opn4. More recently, another laboratory replicated the protocol using mouse iPSCs to first differentiate into RPCs before further differentiating into photoreceptors. After 7 days in differentiation medium, the Pax6-transfected cells express nestin, Tubb3, Nefm, Brn3, and cone–rod homebox (CRX), indicating that the protocol produced a mixed population of retinal cell types including putative RGCs.

Furthermore, overexpression of Atoh7/Math5 was reported to induce RGC differentiation in mouse iPSCs. Atoh7/Math5 is a downstream effector of
Pax6,42 and functions to specifies production of RGCs from a subpopulation of RPCs.43,85–88 Math5 overexpression increases the efficiency of RGC generation from mouse iPSCs with Dkk1 and noggin.66 Transient overexpression of Math5 in mouse iPSCs results in upregulation of Brn3b, Islet-1, and Thy1.2 mRNA, while DAPT treatment of these cells further increases expression of Brn3b and Thy1.2 mRNA.66 Immunocytochemistry revealed cells positive for the markers Atoh7 (15%), Brn3b (10%), Thy1.2 (5%), Isll and synaptophysin (Syp) in differentiated mouse iPSCs treated with Dkk1, noggin, and DAPT.66 These results suggest that Math5-overexpression promotes expression of other regulatory genes for RGC specification. Replication of this study in a human PSC line would be beneficial to determine if similar developmental patterns occur since Atoh7 expression appears to be conserved between species in the retina.85

Finally, a previous report demonstrated that Rax-overexpression in mouse ESCs promoted neural differentiation when cocultured with mouse retina explants.31 Cells dissociated from mouse ESC-derived EBs overexpressing Rax and treated with retinoic acid integrated into the inner neuron layer yet were negative for the markers Isll, a marker for RGC, amacrine and bipolar cell, or ELAV-like neuronspecific RNA binding protein 3 (Elav3 also known as HUC), and Elav4 (also known as HUD) both RGC and amacrine markers, indicating that constitute overexpression of Rax and EB differentiation was not successful in generating amacrine or biopolar cells.31 However, cells derived from mouse ESC-derived EBs overexpressing Rax within the ganglion cell layer were positive for Isll indicating differentiation into ganglion cells.31

RGC Differentiation by 3D Self-Organization

An emerging method in stem cell research that may be applicable to RGC generation in vitro is differentiation by three-dimensional (3D) self-organization.76,89–91 In this technique, optic cup formation is driven by self-organization, as it spontaneously occurs via an intrinsic program of cellular interactions with minimum addition of extrinsic growth factors. A previous study by Eiraku et al.89 showed that matrigel and a low concentration of serum replacement directs EBs from mouse ESCs to intrinsically self-organize and pattern to form an optic cup. The resulting optic cup is able to self-evaginate and invaginate in vitro, forming layers including the neural retina. The derived 3D neural retina was shown to intrinsically pattern into cell layers similar to those that arise during normal development.89 Recently, this optic cup 3D self-organizational protocol was repeated in human ESCs.90 As RGCs arise within the neural retina in vivo, this technique may overcome some of the complex coculturing requirements for RGC specification and expansion in vitro which are discussed below.

Enrichment and Purification of RGCs In Vitro

To date, many protocols for RGC differentiation yield low cell numbers and heterogeneous populations of mixed cell types. In this regard, coculture with other retinal cell types may improve the maintenance of RGCs in vitro. These issues relate to the strict modulation of RGC generation and apoptosis that occurs during retinogenesis82 and to neurotrophic support required from neighboring cells for RGC maintenance.93 However, many biochemical or cellular assays require homogenous population of RGCs, thus method to isolate and enrich stem cell–derived RGCs from the heterogeneous population of differentiated cells is desirable. One selection method used with mouse ESCs transfected with Pax6 cDNA involves antibiotic selection of Pax6-positive cells.68,70 This approach involves selection of G418-antibiotic resistant cells (Pax6 expression construct contains a neomycin resistance gene) followed by limiting-dilution culture to preferentially culture cells expressing the RPC transcription factor Pax6. After antibiotic selection, a clonal efficiency of 1% to 2% Pax6-positive cells was reported.70 From a total of 56 isolated clones, only eight were maintained and characterized for differentiation.70 Clones were preferentially selected if they express the NS/PC markers nestin and Msi168,70 or RPC markers Six3 and Vsx2.70 Despite a poor clonal efficiency following limiting dilution, the eight characterized clones differentiated into several retinal types, including RGCs, confirmed by expression of Shh, Isll, Thy1, and Brn3b mRNA, and Tubb3, Nefm, and Brn3a protein markers.70 It remains to be determined whether isolation of PSCs expressing endogenous levels of RPC regulatory proteins such as Pax6 followed by a defined differentiation protocol will be a useful approach for enriching for RGCs.

Another method to isolate RGC-like cells from primary cell cultures is immune-sorting using cell...
surface marker. Immunopanning is a technique involving antibody-mediated plate adhesion of cells positive for a specific surface antigen. First pioneered in rat retina, RGC-like cells were successfully isolated by a two-step immunopanning (TSI) procedure with the use of two monoclonal antibodies to the Thy1 antigen.\textsuperscript{51} The outcome was a yield of 25\% to 50\% RGCs; however, other cell types including glia and macrophages, are known to also express the Thy1 antigen, thus this method can be variable in success.\textsuperscript{51} When applied to primary cells from human fetal retinas, the TSI procedure successfully isolated RGCs expressing Thy1. In addition to TSI, other purification techniques have been developed. One study compared three isolation techniques: the TSI immunopanning method, direct magnetic sorting, and immunopanning-magnetic separation (IMS).\textsuperscript{52} The DMS protocol applied microbeads coupled with antibodies to isolate cells that are positive for Thy1 expression,\textsuperscript{52} while the IMS method involved immunopanning followed by DMS.\textsuperscript{52} Immunocytochemistry of cells positive for Thy1 and negative for glial fibrillary acidic protein (GFAP) and amacrine cell-specific syntaxin-1 was used to determine the purity of RGC-like cells from each of these methods. Purities of the derived RGCs were reported to be 94\% for immunopanning, approximately 62\% for DMS and approximately 98\% for IMS.\textsuperscript{52} Thus, the DMS technique resulted in significantly lower RGC purity as compared with the other two methods for isolation.\textsuperscript{52} Although the number of surviving RGCs from these isolation methods was not reported, this study highlights the feasibility of TSI and IMS for isolating RGC from primary cell cultures. While the total yield in isolated cells from these isolation procedures is somewhat variable, it is feasible that the immunopanning technique could be used to extract cells expressing more than one RGC-specific surface antigen by serial immunopanning isolations to purify RGCs from mixed population of retinal cultures differentiated from PSCs.

**Perspectives and Conclusions**

This review highlights the growing field of research surrounding in vitro retinal differentiation, with particular focus on RGC generation for modelling optic neuropathies such as glaucoma. Although many advances have been made in this exciting new field it is apparent that the culture and generation of RGCs in vitro is an ominous task. While an ominous task, primary RGCs can be isolated from retinal tissues and potentially used toward transplantation, however, this cannot be a viable therapeutic option for patients with glaucoma due to the limited source of such primary tissues that must be acquired postmortem as well as risks for immunorejection following transplantation into the patient. In this regard, iPSCs offer a unique source of cells to generate immunologically matched RGCs and hold potential for clinical translation for two reasons: firstly, iPSCs are capable of proliferating indefinitely in vitro, therefore could potentially serve as an unlimited cellular source for RGCs for research and clinical applications. Secondly, iPSCs can be generated directly from an affected patient, thereby minimizing the risk of immunorejection following transplantation. Until cell-replacement therapy is available for glaucoma, the PSC field is well poised for in vitro disease modelling to understand the pathogenesis of the RGC loss. As summarized here, RGCs can be derived from PSCs by directed differentiation using growth factors/small molecules, genetic modification, or 3D self-organization. Subsequently, PSC-derived RGCs can be enriched and purified using different methods of antibody-based separation techniques. While iPSCs provide an unlimited supply of cells from which to derive RGCs, current challenges include low efficiencies and purities of RGCs. Future research to address these problems will be critical for generation of an in vitro RGC system for disease modelling as well as for development of cell therapies to treat optic neuropathies such as glaucoma.

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References

29. Voronina VA, et al. Mutations in the human RAX homeobox gene in a patient with anoph-


88. Brzezinski J, et al. Math5 defines the ganglion cell competence state in a subpopulation of retinal


