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Human Pluripotent Stem Cell-Derived Retinal Ganglion Cells: Applications for the Study and Treatment of Optic Neuropathies

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Serving to connect the eye with the brain, retinal ganglion cells (RGCs) play a critical role in transmitting visual information in the central nervous system. As such, damage to or degeneration of these important cells often results in visual impairment and eventually blindness. While few treatment options exists to repair damaged or degenerating RGCs, efforts have often focused upon the restoration of existing RGCs through approaches to encourage enhanced survival of these cells as well as efforts to promote axonal regeneration[1-4]. However, once a critical number of RGCs have been lost, it will likely be necessary to identify a suitable source of cells for replacement purposes, and design strategies to encourage proper survival and integration of cells replacement cells.

Human pluripotent stem cells (hPSCs), including both embryonic and induced pluripotent stem cells, have received considerable attention in recent years for their potential to serve as an effective *in vitro* model of retinal development and repair[5-14]. Furthermore, RGCs differentiated from hPSCs have also been proposed as a powerful, unlimited source of cells for neuroprotective and cell replacement applications[15-17]. However, to date there exist no effective therapies for RGC damage or degeneration and in order for the potential of hPSCs to be realized for such applications, a number of obstacles exist which must be overcome.

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Compliance with Ethics Guidelines

Conflict of Interest Statement

THE AUTHORS NEED TO SUBMIT THEIR DISCLOSURE FORMS

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This article contains no studies with human or animal subjects performed by the author.

Degeneration and damage of RGCs

Damage to RGCs, including the eventual loss of these cells, is characteristic of optic neuropathies, often resulting in the severing of communication between the eye and the brain[18, 19]. While glaucoma is the most common of the optic neuropathies with a current incidence of greater than 60 million people worldwide[20, 21], the optic neuropathies include a variety of other disorders including Leber's Hereditary Optic Neuropathy (LHON) and ischemic optic neuropathies[22-24]. The end result of each of the optic neuropathies is the loss of RGCs, but the underlying causes are many and varied. As such, the development of therapeutic approaches for the treatment of optic neuropathies has also been varied. The preservation of existing RGCs is often viewed as the most promising approach for the development of treatments for optic neuropathies[22-24], due to the fact that existing cells are at least partially integrated into the retinal circuitry. In many forms of glaucoma, an increase in intraocular pressure (IOP) is thought to serve as a trigger resulting in damage to RGCs, and eventually leading to the loss of these cells[25-27]. As such, a common existing treatment for glaucomatous neurodegeneration involves the reduction of IOP with interventions including eye drops and laser treatments being among the most common approaches[28-31]. These approaches to lower IOP have been successful in delaying glaucomatous neurodegeneration and prolonging vision, but do not prevent the subsequent damage to, and loss of, RGCs. This eventual loss of RGCs is due to the fact that these interventions to lower IOP have just transient effects, while elevated IOP will persist beyond the treatment period. Furthermore, fluctuations in IOP levels are known to occur, particularly those diurnal in nature[32-34]. Thus, existing interventions may not effectively prevent subsequent damage to RGCs as IOP levels are known to fluctuate.

Due to the transient nature of interventions to lower IOP, additional strategies to rescue RGCs and prevent their eventual loss have been a popular topic of research for a number of years. To this end, pharmacological approaches have been particularly attractive for the development of neuroprotective strategies. In particular, neurotrophic factors have received considerable attention for their potential ability to delay or halt neurodegeneration. Within the optic neuropathies, neurotrophic factors including brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), and ciliary neurotrophic factor (CNTF) have been the subject of numerous studies due to their documented ability to prevent apoptosis and prolong the viability and functionality of RGCs[35-37]. While these neurotrophic factors have been successful in this capacity, this success has been transient in nature as these protective effects do not persist for prolonged periods of time and eventually, RGCs suffer damage and are lost. While the reasons for this transient nature of neurotrophic factors and their effects on RGCs are likely varied, research has demonstrated that RGCs tend to become acclimated to elevated levels of these neurotrophic factors and become desensitized to their effects, often through the downregulation of specific receptors[38-40]. Thus, further examination of factors that may aid in the neuroprotection of RGCs is warranted, with the goal of identifying those factors that may confer a long-lasting neuroprotective effect and successfully rescue the degenerative phenotype of optic neuropathies.

Stem Cells and Retinal Ganglion Cells

The ability to derive RGCs from stem cells offers numerous exciting opportunities for investigation, including the underlying mechanisms responsible for their damage and loss in optic neuropathies. Furthermore, stem cell-derived RGCs can also be developed as a tool for cell replacement following the degeneration and loss of endogenous RGCs. A variety of types of stem cells exist, each with specific properties and characteristics that enable their use for a variety of applications, both basic and translational in nature. In recent years, however, the ability to derive retinal neurons from pluripotent stem cells has received considerable attention and is the subject of ongoing investigation[8-13, 41-49]. Human pluripotent stem cells (hPSCs), including both human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs), offer numerous advantages for the development of RGC-related approaches. First, hPSCs can be expanded indefinitely *in vitro*, thus possessing the potential to provide an unlimited source of cells for any application. Additionally, unlike other stem cell sources, hPSCs possess the innate ability to differentiate into any cell type of the body, including RGCs, offering unprecedented access into the developmental mechanisms underlying RGC differentiation, as well as those characteristics within RGCs that may be responsible for diseases that affect RGCs. Furthermore, when derived from a patient-specific source, hiPSCs can serve as effective *in vitro* models of retinal disease progression(9, 48, 50, 51), and may provide an unlimited source of autologous cells for cell replacement applications following the loss of endogenous retinal cells[5-7, 14, 52, 53].

Within the past decade, a number of studies have demonstrated the feasibility of deriving retinal cells from hPSCs[7-13, 16, 42-49]. Early studies documented this ability in hESCs but upon their discovery in 2007[54-57], studies quickly documented the similar ability of hiPSCs to yield cells of the retinal lineage as well[9, 10, 13, 16, 42, 58]. These early efforts relied upon the treatment of differentiating hPSCs with specific signaling molecules. Interestingly, variation in the types of signaling molecules required for retinal differentiation was initially reported by a number of groups[8, 12, 13, 42, 58]. Despite these different approaches, the similar differentiation of retinal neurons was likely due to an innate, default ability of hPSCs to differentiate toward the neural lineage, with the retinal lineage among the more common neural lineages generated. Consequently, studies in recent years have developed and refined retinal differentiation protocols from hPSCs to minimize or eliminate the use of exogenous signaling factors, instead relying upon the innate ability of hPSCs to yield a retinal fate[9, 10, 43, 44, 46]. This innate retinal differentiation capacity has been further exploited to induce hPSCs to not only differentiate into retinal cell types, but to do so in a manner which recapitulates the three-dimensional differentiation and organization of retinal cells into a retinal-like tissue[11, 45, 49].

While many studies have demonstrated the ability to derive retinal cells from hPSCs, the demonstration of yielding RGCs from hPSCs has been less extensively studied. Most studies to date have focused on the differentiation of photoreceptors and retinal pigment epithelium from hPSCs[5-10, 12-14, 42, 52, 58-60], likely due in part to the number of prevalence of blinding disorders that adversely affect these cell types. Just as important, however, is the fact that photoreceptors and retinal pigment epithelium cells can be identified *in vitro* due to the existence of specific markers that can be utilized to identify these cell types. The

definitive identification of RGCs, however, proves to be more difficult. While some markers can definitely identify RGCs within the retina itself, these markers are not truly specific to RGCs. When derived from hPSCs, the pluripotent nature of these cells requires careful consideration about the specificity of markers utilized to identify differentiated cell types. The definitive identification of RGCs from a pluripotent source necessitates that a number of criteria are met (Table 1). First, differentiated hPSCs should express markers characteristic of RGCs, including commonly identified markers such as Brn3, Islet1, Pax6, etc. Additionally, these prospective RGCs should possess characteristic morphological features. In particular, RGCs are unique within the retina due to the extensive outgrowth and elongation of axonal projections, as well as the existence of a significant number of dendritic extensions[61-64]. Similarly, presumptive hPSC-derived RGCs should possess the ability to develop comparable morphological characteristics. Furthermore, due to the fact that many of these features are shared with some other non-retinal neuronal populations of the central nervous system[65, 66], care should be taken to demonstrate the retinal lineage of presumptive hPSC-derived RGCs, particularly through the stepwise, documented differentiation of RGCs through all of the major stages of retinogenesis. Finally, upon the acquisition of the above phenotypic characteristics of RGCs, the proper physiological activity of these cells would need to be documented[67, 68]. As RGCs *in vivo* are responsible for conducting neural information from the eye to the brain, the functionality of hPSC-derived RGCs requires their physiological activity.

Given the above criteria necessary for the identification of RGCs from a pluripotent source, it is not surprising that a general lack of studies exist focused upon this topic. However, the potential for hPSCs to yield RGC-like cells has received increased attention in recent years. While not necessarily focused upon the specific differentiation of RGCs, a number of groups have documented the expression of some RGC-associated markers in the process of differentiating retinal cells from hPSCs (Table 2), particularly the expression of the transcription factor Brn3[9, 11, 42, 44-49, 69-71]. Advancing features of RGC differentiation have been documented less frequently, although more recent studies have demonstrated the development and extension of axonal-like structures from presumptive hPSC-derived RGCs, demonstrating the ability of these cells to develop RGC-related morphological features[9, 44, 69-71]. Finally, among the most recent studies, efforts have been focused on demonstrating some degree of physiological activity from presumptive RGCs, including the elicitation of action potentials[70, 71].

As efforts progress in the coming years, it is likely that the ability to derive RGCs from hPSCs will become standardized and more routine. Once this occurs, the potential will exist for the translational application of these differentiated cells. The ability to utilize patient-derived hiPSCs as an *in vitro* model of human disease will likely be among the first applications to realize this potential. When hiPSCs are generated from specific patient samples, particularly those with a genetic basis underlying a disease phenotype, the successful differentiation of the affected cell type can serve as an effective model of disease progression[72, 73]. Such an ability has been demonstrated in a variety of other systems, including other cell types of the retina including photoreceptors and retinal pigment epithelium[9, 16, 51], although similar efforts to model optic neuropathies *in vitro* have been

limited to date. Recently, Tucker et al[48] examined the role of autophagy in RGC-like cells derived from hiPSCs that were generated from a patient with a form of normal tension glaucoma, with the demonstration that patient hiPSC-derived RGCs exhibited differences in LC3 expression associated with autophagy deficits. Similarly, Minegishi et al[74] demonstrated the ability to derive hiPSCs from a patient with a related form of normal tension glaucoma. Although the differentiation of these hiPSCs to an RGC fate was not demonstrated, these cells did demonstrate various features associated with the progression of normal tension glaucoma. Thus, while tremendous potential remains for hiPSCs to serve as an effective model of disease progression, preliminary studies demonstrate the feasibility of such an approach.

Along with the potential for hiPSC-derived RGCs to act as an *in vitro* disese model, they can also serve as a unique tool for drug development purposes[75-77]. Due to the ability to obtain unlimited numbers of hiPSCs, along with the ability to direct the differentiation of these cells to a retinal lineage (including RGCs), the potential exists to test the efficacy of novel compounds upon diseased cell types for their therapeutic potential. Such an ability to utilize hiPSCs for drug screening purposes has been preliminarily demonstrated in a variety of other system to date, including within the retina[9, 16, 51]. In order for this potential to be realized for RGCs, a clearly identifiable and measurable disease-related phenotype must exist by which to test the efficacy of such compounds. Given this ability, the potential for high-throughput screening approaches exists as well, potentially expediting or enabling the development of new drugs for optic neuropathies.

Beyond the applications of hiPSC-derived RGCs *in vitro*, the potential exists for the development of cellular replacement approaches, particularly following the loss of RGCs in response to optic neuropathies or other related injuries. Cellular replacement with hPSCs is an attractive option for future therapeutic approaches to optic neuropathies, particularly at later stages of the disease process. While pharmacological intervention may be effective at early stages of disease progression, cellular replacement becomes the only viable option once significant cell loss has occurred. The potential for cellular replacement with hPSCs has been demonstrated in preliminary studies typically in rodent models, particularly including cells of the outer layers of the retina such as retinal pigment epithelium and photoreceptors[5-7, 14, 52]. The successful replacement of RGCs, however, will likely be more difficult due to certain characteristics of RGCs, particularly their need to extend axons across long distances. True replacement of RGCs will need to demonstrate that a number of criteria are met. First, transplanted cells will need to integrate into the appropriate location in the retina, namely the retinal ganglion cell layer. Following successful integration, these cells will need to extend axons into the nerve fiber layer, enter the optic nerve and extend further into the visual areas of the brain. This axonal elongation also requires the proper ipsilateral or contralateral projection into the brain, particularly in humans and other primates[78, 79]. If these requirements are met, axons must match with appropriate post-synaptic targets and establish functional connections, and axons need to be myelinated to properly convey neural impulses. Beyond all of the requirements for axonal elongation and synaptogenesis, similar processes must also occur in RGC dendrites to receive synaptic input and functionally integrate into the retinal network. Finally, transplanted cells would

need to be capable of surviving the local microenvironment, which is often highly toxic to RGCs and resulted in the original loss of host cells in the first place[80]. Thus, while the potential exists for hPSC-derived RGCs to serve as a tool for cellular replacement approaches, their complex and unique nature within the retina also serves to complicate efforts to replace them once they have been lost due to disease-related processes.

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Table 1

Degree of RGC Maturation

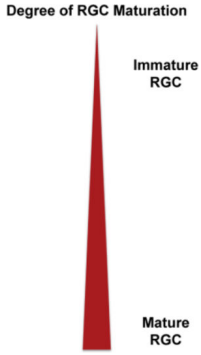
Property	Specific Criteria	Degree of RGC Maturation
Neuronal morphology	Complex neurite outgrowth	
Expression of RGC markers	BRN3, ISL1, HuC/D, PAX6, RBPMS	
Retinal lineage derivation	Differentiated through retinal progenitor intermediary Expressed in association with other retinal markers Lack of expression of markers indicating lineages with similar markers	
Neuronal Polarization	Compartmentalization of axons and dendrites Distinction of Tau and MAP2 immunostaining	
Electrophysiologically active	Hyperpolarized resting membrane potential Voltage-gated ionic currents Elicitation of action potentials	

Table 2

Demonstrated ability to derive RGC-like cells from hPSCs

Markers:	**Functionality:**	**Application:**
BRN3 TUJ1 (BIII-TUBULIN)	N/A	N/A
BRN3	N/A	N/A
BRN3 TUJ1 (BIII-TUBULIN)	N/A	N/A
BRN3 MAP2 ISLET1	N/A	N/A
BRN3	N/A	N/A
BRN3 HU C/D	N/A	N/A
BRN3a BRN3b TUJ1 (BIII-TUBULIN)	N/A	N/A
BRN3 TUJ1 (BIII-TUBULIN) HU C/D MAP2	N/A	N/A
BRN3 γ-SYNUCLEIN ISLET-1 THY-1 BRN3b MATH5	Resting potential of −50mV Ability to evoke EPSC	None
BRN3b TUJ1 (BIII-TUBULIN) γ-SYNUCLEIN ISLET-1 MATH5 TAU NFL NFM	Resting potential of −80mV Able to evoke EPSC	None
BRN3b TUJ1 (BIII-TUBULIN) MAP2 THY-1 NF200 ATOH7	None	hiPSCs disease model normal tension glaucoma (TBK1 gene duplication)