IN VITRO GENERATION OF HUMAN RETINAL GANGLION CELLS VIA DIRECT CONVERSION AND STEM CELL DIFFERENTIATION

by

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Abstract

Retinal ganglion cells (RGCs) are essential for human visual perception, as they mediate the transfer of visual information from the eye to the brain. In the event of RGC death, as happens in diseases such as glaucoma, vision is permanently lost because the mammalian central nervous system (CNS) does not regenerate. In order to find novel treatments for the RGC diseases, a number of different research approaches have been undertaken. One relatively recent approach involves the use of pluripotent stem cell (PSC) technologies. As human PSCs can be differentiated to the RGC lineage, it has become possible to utilize human RGCs for drug discovery, disease modeling, or transplantation experiments to attempt visual system recovery.

The field of PSC differentiation to RGCs has been steadily developing, but many hurdles remain. Initial reports involved co-culture of mouse stem cells with primary embryonic retinal cells or conditioned media, and yielded only small numbers of cells. Evolution of ocular stem cell biology over the past few years has greatly improved the technology for generation of three-dimensional optic vesicles that develop all of the retinal layers, including the RGC layer. Although such reports were very encouraging, these studies failed to demonstrate that RGCs could be isolated from culture and deeper profiles of the RGCs were missing. Here, we describe a novel, simplified protocol for RGC differentiation from human PSCs. We took advantage of recently developed CRISPR-Cas9 technologies to genetically engineer a stem cell reporter line for RGCs based on the BRN3B/POU4F2 gene. Using this line, we developed a fluorescence-activated cell sorting (FACS) protocol that yields highly purified RGCs, and we
subsequently characterized the purified cells in terms of their expression pattern of RGC-associated genes and other cellular properties. Despite the power of FACS to provide purified populations of fluorescent RGCs, FACS-based purification schemes also have their limitations. A main limitation is the relatively slow speed and restricted throughput of FACS. In order to get around these limitations, we developed an alternative approach by genetically engineering a unique cell surface antigen driven by the *BRN3B* gene into stem cells, allowing us to isolate populations of pure differentiated RGCs by affinity purification in an efficient, large scale, and time saving manner that makes possible the use of human RGCs in a variety of studies including high throughput drug discovery screens. In addition to developing this new RGC purification strategy, which has implications for developing improved systems for the purification of a wide variety of different cell types, we have also optimized our initial differentiation protocol by manipulating known signaling pathways via small molecule supplementation to guide the cells toward a retinal cell fate.

In addition to developing and characterizing improved stem cell-based approaches for generating RGCs, we have also been pursuing the generation of RGCs from non-stem cell lines through trans-differentiation and direct reprogramming. Through this work, we have identified a combination of four transcription factors that can directly reprogram the human retinal pigment epithelium (RPE) cell line ARPE19 into RGC-like cells. Expression of *ATOH7*, *BRN2*, *BRN3B*, and *MYT1* in ARPE19 cells transformed their morphology to a neuronal phenotype and induced the expression of pan-neuronal and RGC-associated genes. Moreover, when overexpressed in stem cells undergoing retinal differentiation, these factors were able to boost the percentage of cells differentiating to
the RGC lineage, an effect that could be further increased by our previously identified small molecules.

Lastly, we discuss the development of a new method to enhance homology directed repair for the purpose of generating reporter lines in an easier and more routine way. Taken together, these studies provide powerful tools for the use of stem cell technology to study RGC differentiation and biology, and the mechanisms of RGC injury and cell death. Additionally, they provide the means to provide well-characterized and large supplies of RGCs for drug discovery screens and lay the groundwork for possible future cell-based therapeutic approaches for treatment of vision loss and blindness from glaucoma and other forms of optic nerve disease.
Dissertation Referees

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Chapter 1: Introduction


1.1 The retina and glaucoma

Visual perception by the brain begins in the retina, the light sensing neural tissue located in the back of the eye. This tissue is organized into specific layers of cells and consists of seven major cell types: photoreceptors (rods and cones), horizontal cells, bipolar cells, amacrine cells, retinal ganglion cells (RGCs), and Müller glia. Light perception begins with photoreceptors, these cells undergo hyperpolarization in response to light, thus converting light into an electrical signal that is then propagated to the rest of the retinal neurons for further processing. Once this signal reaches the RGCs, it is sent to the brain via a bundle of RGC axons, i.e. the optic nerve. Damage to the retina can result in the death of one or more of these retinal cell types, thus leading to vision loss. Most retinal degenerative disorders result in the death of photoreceptors or RGCs, leading to irreversible blindness since these cells do not regenerate. One of the most common retinal degenerative disorders is glaucoma, an optic neuropathy marked by axonal damage and progressive loss of RGCs, and therefore, vision¹. Unfortunately, due to the gradual loss of vision in glaucoma, patients can be unaware that they even have the disease until significant RGC loss has already occurred. Glaucoma is a multifactorial disorder, with increased intraocular pressure (IOP) being the best characterized risk factor, and the only one that is currently amenable to treatment². IOP can be lowered with eye drops, laser treatment, and other forms of surgery. However, lowered IOP does not always prevent further degeneration, and glaucomatous optic nerve damage can arise and progress even in individuals who have
never had documented increased IOP. Thus, there is a need to develop additional therapeutic strategies, beyond IOP lowering, to slow down, and prevent progressive RGC damage in glaucoma. One such approach is neuroprotection\textsuperscript{3-5}. Neuroprotection focuses on improving survival and function of neurons after the initial neuronal insult. Additionally, there is a need to develop approaches to help restore vision for patients who have already lost too many RGCs.

In order to develop novel therapeutic strategies for glaucoma and other retinal neurodegenerative diseases, cell-based drug screening can be utilized to identify neuroprotective molecules. Generally, to procure cells for such screens, primary RGCs are isolated from rodent retinas. Primary rodent cells are easily accessible and they can represent \textit{in vivo} biology. However, they become damaged during the isolation/purification procedure and generally die quickly as a result. As an alternative approach, \textit{in vitro} generated RGCs are not damaged in this manner and survive for long periods in culture. Additionally, human RGCs can be generated \textit{in vitro}, allowing for access to human biology as well as patient specific biology, thus moving closer to modeling of human disease in a dish. In order to generate RGCs \textit{in vitro}, pluripotent stem cells (PSCs) can be differentiated or somatic cells can be directly reprogrammed or converted with transcription factors to transdifferentiate to an RGC cell fate.

\subsection*{1.2 Stem cells}

Since their discovery, embryonic stem cells (ESCs), and the subsequently discovered induced pluripotent stem cells (iPSCs), have held great promise for regenerative medicine, disease modeling, and the study of developmental biology \textit{in vitro}. As these cells
are pluripotent, and able to differentiate into any cell type in the body, RGCs can be generated from stem cells to be used for studies of the mechanisms of RGC injury and cell death, drug discovery, and development of cell-based therapies for glaucoma and other forms of optic nerve disease, including RGC transplantation to replace lost cells.

When PSCs differentiate, they appear to follow the natural developmental process. In order to preserve their undifferentiated state, PSCs require daily changes of media consisting of multiple components, most importantly FGF2 and TGF-β proteins, which the cells rely on to maintain pluripotency\textsuperscript{6-8}. If neglected, these cells differentiate nonspecifically, and they tend to differentiate into a highly heterogeneous mixture of cell types as highlighted by the propensity of ESCs to form teratomas, tumors consisting of all three germ layers, when injected into immunodeficient mice\textsuperscript{9}. However, using knowledge gained from developmental studies as well as empirical experimentation, researchers have been able to modify culture conditions in order to direct stem cell differentiation down a particular path. In this manner, by modulating culture conditions to favor one tissue or cell type over another via addition of agonists/inhibitors of specific signaling pathways, more homogenous populations of cells can be generated that contain, for example, neurons, cardiomyocytes, hepatocytes, or hematopoietic cells. In addition to signaling molecules, cells also physically interact with each other as well as their environment, highlighting the need for appropriate extracellular matrix components in order to generate the desired cell type\textsuperscript{10}. Furthermore, through genome editing or transgene delivery, stem cells can be programmed to overexpress lineage specific genes at predetermined time points in order to direct differentiation. Taken together, stem cell differentiation from ESCs remains an
active field of research with expanding protocols that build on previous studies to improve the process to yield purer and better defined populations of cells.

Despite the immense potential that human ESCs hold for the future of medicine, they also continue to be subjected to ethical concerns regarding their embryonic origin. The question of whether a human embryo should be used for scientific discovery or indirectly for medical treatment is a personal question for many, and the direct effect is felt by the academic community as collaborations between groups can be hindered due to hESC use in experiments, especially between international groups that abide by prohibitive laws. The discovery of iPSCs has reduced the demand for utilization of embryos for research\textsuperscript{11} and these cells have become the stem cell model of choice for a large number of labs.

iPSCs can be generated from somatic cells, such as fibroblasts, through the overexpression of four transcription factors. Initially, four necessary genes (Oct-3/4, Sox2, c-Myc, and Klf4) were delivered to cells via retroviruses. More recently, however, it has been shown that non-viral and non-genome integrating methods of gene delivery can also be used for the generation of iPSCs\textsuperscript{12}. On a functional level, like their ESC counterparts, iPSCs are capable of differentiating into any other cell type in the body using the same ESC differentiation protocols. Moreover, on the genetic and protein levels, iPSCs and ESCs are generally similar, and mouse iPSCs have been used to create fully formed mice\textsuperscript{13}. Additionally, human iPSCs offer the distinct advantage of modeling patient diseases \textit{in vitro} and providing patients with the possibility of personalized studies designed to identify the most promising treatment strategy for their particular form of the disease. Through the collection of skin biopsies, a patient's cells can be used to make
iPSCs that could be expanded in culture and used to generate cell types of medical interest. In this way, a patient with glaucoma could donate a small amount of skin tissue that would be reprogrammed into iPSCs and then differentiated to RGCs that could be used for drug screening to find the optimized glaucoma treatment for that particular patient.

Stem cells may be used for glaucoma research and/or treatment in multiple ways. As stated previously, stem cell differentiation to RGCs may provide a robust in vitro system for generating a medically relevant cell model for drug discovery. Additionally, stem cells themselves may also be used as a means of cell therapy. For example, part of glaucoma pathophysiology is thought to involve the loss of retrograde neurotrophic transport to the RGC soma from damage to the optic nerve and/or increased IOP\textsuperscript{14-16}. One possible approach to this problem is exogenous supplementation of neurotrophic factors (NTFs) to the retina in an effort to promote RGC function and survival. NTFs such as brain-derived neurotrophic factor (BDNF), ciliary-neurotrophic factor (CNTF), and glial cell-derived neurotrophic factor (GDNF) all promote RGC survival in vitro and in vivo, but their delivery to the retina, and specifically RGCs, is problematic\textsuperscript{17-20}. These NTFs would need to be administered in relatively high doses to have a positive effect, but they cannot be taken orally because the blood-retinal barrier and other factors would likely prevent sufficient delivery to the retina. Topical eye drops also would likely not provide sufficient NTF delivery to the retina as well. Local injection of NTFs into the vitreous is technically possible, and could provide a benefit, especially if combined with a slow-release formulation. However, such an approach would still involve fairly frequent injections over a prolonged period and, due to inconvenience, expense, and the risk of
infection, would not be ideal. Stem cells could provide an alternative and more desirable approach. One approach is to use mesenchymal stem cells (MSCs) which are derived from the bone marrow of adult patients and are known to secrete a number of NTFs. Another exciting possibility is the implantation of encapsulated cells into the eye to allow for continued release of supporting proteins. Cellular encapsulation allows nutrients to enter the implant, and the implant materials can be designed so that only proteins of a desired size are able to leave the implant. Thus, through selective permeability, the implanted cells should be able to safely provide a long-term supply of therapeutic proteins, growth factors, or antibodies targeting particular pathways or cellular components.

Encapsulation of cells is also applicable to PSC-derived cells. PSCs could be differentiated to NTF-producing cells prior to encapsulation and then implanted into the vitreous of the eye. Moreover, PSC-derived cells used in cell therapy may provide a better alternative to MSCs. For example, if human iPSCs were differentiated to retinal cells prior to encapsulation, the implanted cells would not be foreign to the retina or the patient and would likely be much less immunogenic. Additionally, if post-mitotic retinal cells were used for the implant, (e.g. RPE), then the chance of implant rupture due to cell division would also be minimized. As another potential safety valve, implants could be removed or replaced at a later time as needed. Cell therapy is appealing because it allows for sustained treatment through protein supplementation, leading to a decreased need for multiple injections of therapeutic factors.
1.3 RGC genetics and development

While the stem cell field continues to increase the portfolio of possible-to-generate cell types and tissues, the list is still incomplete. However, there is much excitement in the retinal field since despite early struggles, stem cell differentiation to the retinal cell fate has been demonstrated, with some very impressive reports describing the formation of whole retinal tissues that contain RGCs\(^{24,25}\). When ascribing the term of “RGC” to some of the differentiated cells found in these cultures, it is important to consider what makes this cell type unique and how can we be sure of its identity. In the context of an \textit{in vivo} developed retina, there is good agreement regarding the definition of an RGC, but outside the retina, separated from their normal location and synaptic connections, the definition of what is an RGC becomes much less clear. Compared to other types of neurons, and especially developing neurons in culture, RGCs do not have a unique morphological appearance. There are no known RNAs or proteins whose expression is strictly limited to RGCs, nor are there electrophysiological properties that can distinguish them from many other neurons. Additionally, there is also some disagreement in terms of how many different types of RGCs exist, with some defining greater than 20 different subtypes\(^{26}\). Since many of these cell types lack known specific genetic signatures and are defined by morphology or electrophysiological visual responses, it tends to be even more difficult to classify \textit{in vitro} generated RGCs according to their subtype. However, if we focus on the more broad RGC cell class, then we can move closer to a working definition of an RGC. Certainly, rather than looking at just a few RGC-enriched genes as markers, it would be more meaningful to take a more systems oriented view and to use a larger group of genes whose overall pattern of
expression better define RGC identity. However, even this aspect is challenging, as the
gene expression pattern of RGCs will vary to a greater or lesser extent between different
subclasses and perhaps stages of development and degeneration. Without careful
consideration, one might confuse stem cell-derived RGC-like cells with other neuronal
cells such as motor or auditory neurons. The robust characterization of stem cell-derived
RGCs may also contribute to developmental research by highlighting similarities and
differences between in vitro versus in vivo and human versus non-human development.

In an effort to narrow down an RGC-specific genetic signature, it is important to
consider their developmental source. RGCs arise from common retinal progenitor cells
(RPCs) that are the precursors of all retinal cell types. Despite considerable overlap in the
temporal formation of different retinal cell types, RGCs are the first cell type born from
RPCs, followed by horizontal cells, cone photoreceptors, amacrine cells, rod
photoreceptors, bipolar cells, and Müller glia. During development, RGCs are
overproduced, and only those that find an appropriate target in the brain survive through
a process that is thought to involve the retrograde transport of NTFs from the brain to the
retina\textsuperscript{14,28}. Many RGCs die during this process via apoptosis, or programmed cell death\textsuperscript{29}.

Although RGC competence, or the ability for a cell to become an RGC, has been
suggested to arise through a stochastic process\textsuperscript{30}, genetic studies have identified a number
of genes that play a role in the establishment of RGC competence and subsequent control
of RGC differentiation. One of the early active genes in retinal differentiation is \textit{PAX6}.
This gene is required for all retinal cell types except amacrine cells\textsuperscript{31}. It functions
upstream of the \textit{Math5 (ATOH7)} transcription factor, an essential RGC cell fate regulator.
\textit{Math5} is the mouse ortholog of atonal, a basic helix-loop-helix (bHLH) gene found in
Drosophila where it is necessary for photoreceptor formation. In vertebrates, \textit{Math5} plays a bigger role in RGC genesis. It is expressed transiently in the mouse retina, starting at embryonic day-11 (E11), peaking at E13.5, and ending at E15.5\textsuperscript{32}. Its expression pattern overlaps with RGC genesis\textsuperscript{33}, and mice lacking the \textit{Math5} gene have a severe reduction in RGC number (>80%) as well as extremely thin optic nerves\textsuperscript{32,34}. These knockout mice also demonstrate a disruption in their circadian rhythm, likely due to the missing intrinsically photosensitive RGCs (ipRGCs)\textsuperscript{35}. Deletion of \textit{Math5} results in the absence of the RGC-enriched transcription factor \textit{Brn3b}, suggesting that \textit{Brn3b} is downstream of \textit{Math5} in the RGC differentiation pathway. After differentiating from RPCs, 80% of RGCs express \textit{Brn3b}, and shortly thereafter the related transcription factors \textit{Brn3a} and \textit{Brn3c} are turned on in 80% and 20% of RGCs, respectively\textsuperscript{36}. The knockout of \textit{Brn3b} results in a 70% loss of RGCs in adult mice, suggesting that this gene is also important for RGC survival\textsuperscript{37}. The \textit{Isl1} gene is also necessary for RGC survival, as has been shown by the finding that mice in which it has been knocked out generate RGCs normally, but most of these cells undergo apoptosis soon after birth\textsuperscript{38}. The double knockout of \textit{Brn3b} and \textit{Isl1} leads to a more severe RGC phenotype, with approximately 95% death of RGCs, suggesting at least some independence in the pathways in which they function\textsuperscript{39}. Adding to the complexity of this system, it has recently been proposed that a small subset of RGCs form from \textit{Neurod1} expressing RPCs in a pathway independent of \textit{Math5}\textsuperscript{40}. Previously, it was thought that \textit{Math5} functioned, in part, to inhibit \textit{Neurod1}, and that \textit{Neurod1} acted to specify another cell fate entirely\textsuperscript{41}. However, more recently, it has been found that \textit{Neurod1} expressed in the \textit{Math5} locus partially rescued RGC formation\textsuperscript{42}, but the reverse was not true for \textit{Math5} replacement of \textit{Neurod1}, as this exchange led to RGC
formation rather than the photoreceptor and amacrine cells that Neurod1 normally specifies\textsuperscript{43}. These results demonstrate, as is also known from other systems, that not only the nature of the regulator expressed but also its temporal and spatial expression pattern are important in determining the functional effects of a particular transcription factor. Additionally, these studies highlight that although impressive advances have been made in elucidating the factors necessary for RGC competence and cell fate determination, the pathway to RGC differentiation remains an incompletely understood process.

In addition to genes that establish competence, genes important for RGC specification are also a valuable area of research since not all progenitor cells that express Math5 eventually adopt the RGC cell fate, emphasizing that other factors are needed to fully instruct a cell down a particular path. The Sox4 and Sox11 genes appear to be important players in this process as their double knockout results in reduction in RGCs and Brn3b expression, yet these mice possess normal Math5 expression\textsuperscript{44}. More work still needs to be done to elucidate how these genes may function in the differentiation cascade. One way to answer some of these development questions is through PSC differentiation. Since PSCs, in general, mimic the natural developmental state, they should provide a powerful system to study the mechanism of RGC differentiation in the context of a human cell and to complement the elegant differentiation studies that have already been performed in the mouse. We anticipate that the genes discovered through these studies will be useful as RGC markers to test for RGC production \textit{in vitro}. Furthermore, by manipulating this pathway using current knowledge, it may be possible to boost RGC production through over-expression or down-regulation of the already identified genes.
1.4 Stem cell differentiation to RGCs

Stem cell differentiation towards retinal cells has generally focused on production of retinal pigment epithelium and photoreceptor cells. However, since RPCs generate the different retinal cell types in a sequential chronological order\textsuperscript{30}, some of the current stem cell culture systems for producing photoreceptors should, at least at some point during differentiation, also contain RGCs. In fact, the presence of such RGC-like cells has been observed in the past, but a robust protocol for efficient RGC production from PSCs had been lacking until rather recently as will be discussed in the following chapters. Here, we would like to briefly discuss the work of past experimentalists that laid essential groundwork. Some of the early attempts at RGC production were done through ESC co-culture methods with mouse retinas. These studies were based on even earlier work where mouse ESCs (mESCs) grown in suspension were supplemented with Dickkopf-1 (Dkk1), Lefty-A, and Activin-A to favor differentiation to the retinal neuronal fate\textsuperscript{45-48}. To achieve RGC genesis, mESCs were differentiated to an early retinal phenotype before being co-cultured with extracted mouse retinal tissue to further promote a retinal cell fate. Presumed RGC-like cells derived from the ESC cultures were found after 10 days, and a few cells were immunopositive for the RGC-enriched markers Brn3b, Hu, and Tuj1\textsuperscript{49}. The stem cell-derived cells expressed these three markers when found in the \textit{ex vivo} ganglion cell layer of the co-cultured retina. This differentiation was enhanced if the host retina was damaged with N-methyl-D-aspartate (NMDA) prior to co-culture. In a similar study, it was shown that mESCs that were differentiated to the early retina phase could be transplanted directly into a mouse eye\textsuperscript{30}. Moreover, if the retina was again damaged with NMDA prior to transplantation, then RGC-like cells could be readily identified by their
expression of RGC-enriched markers - Tuj1, Brn3b, Pax6, Thy1.2, and NeuN. However, as discussed above, it should be remembered that although within the retina these markers tend to be specific to RGCs, outside the retina they are expressed by a variety of different neurons. Notably, Tuj1 and NeuN are pan-neuronal markers and Thy1.2 is expressed in a large majority of neuronal cells as well. Brn3b and Pax6 are more restricted in their expression pattern, however, and can theoretically narrow the cell type possibilities to RGCs and auditory neurons.  

Co-culture methods such as those described by Aoki and colleagues are challenging because they lack much control over differentiation, introduce potential contamination, and require animal tissue. In an effort to move away from co-culture and toward more defined conditions, an mESC to RGC differentiation protocol was developed that relied on basic fibroblast growth factor (Fgf2) and sonic hedgehog (Shh) supplementation of formed embryoid bodies in neuronal media. Embryoid bodies (EBs) are three-dimensional aggregates of stem cells that form naturally when stem cell colonies are grown in suspension. The cells generated by this protocol expressed some RGC-enriched markers, including Math5, Isl1, and Map2, by immunofluorescence analysis and Math5, Brn3b, Thy1, Rpf-1 (Pou6f2), and Isl1 by RT-PCR. This protocol resulted in roughly 2% of cells that were positive for RGC genes. Functionality of these cells was not explored but transplantation of the RGC-like cells into rat vitreous resulted in some low engraftment into the retina, including the ganglion cell layer. Yet another mouse study showed that Pax6 transfection into mESC cells could force the cells toward a neuronal phenotype and the selection of these cells for further culture resulted in
differentiation to the retinal fate\textsuperscript{53}. Based on RT-PCR, immunofluorescence, and calcium imaging, the authors ascertained these neuronal cells to be RGCs.

Similar differentiation experiments have also been performed using iPSCs. In one study, miPSCs were aggregated into EBs, cultured in the presence of Noggin and Fgf2, and then transferred to laminin and poly-D-lysine coated plates and further cultured in conditioned media from rat embryonic retinal tissues from E14, a time of rat RGC genesis\textsuperscript{54}. These stem cell cultures demonstrated up-regulation of the eye field-related genes \textit{Pax6}, \textit{Chx10}, and \textit{Rx}, and upon growth in conditioned media began to express RGC markers, culminating after one additional month of culture in approximately 15\% of the cells expressing the RGC-enriched markers Brn3b and Rpf1 (Pou6f2)\textsuperscript{55}. Upon culturing of these cells with either superior or inferior mouse colliculus explants, it was encouraging to note that the presumed RGCs extended processes only to the superior colliculus, which is their natural target \textit{in vivo}. Another group attempted to direct differentiation further by supplementing differentiation media with Dkk1, Noggin, and Lefty-A to promote anterior neurogenesis\textsuperscript{56}. To further specify the cells towards an RGC fate, the researchers transfected a \textit{Math5} expression plasmid into the cells prior to starting differentiation and added DAPT, a Notch signaling inhibitor, to the media. The inhibition of Notch was intended to push the cells toward neuronal differentiation\textsuperscript{57}. A subset of RGC enriched genes was indeed induced by this protocol, but less than 5\% of the cells expressed these markers. Taken together, these early studies had shown that differentiation of mESCs and miPSCs towards RGC-like cells was clearly possible, but the reported yield remained low, cultures were heterogeneous, and conditioned media from murine tissues or additional genetic manipulation was often used.
Human stem cells have also been differentiated to the retinal cell fate. One of the first studies\textsuperscript{58} took a similar approach to that described with mESCs. Cells were grown as colonies and lifted up to form EBs that were then cultured in suspension with Dkk1, Noggin, and insulin-like growth factor-1 (IGF-1) before plating and expansion of the cultures on Matrigel, a protein mixture secreted by a mouse sarcoma cell line that mimics an extracellular matrix. This protocol resulted in the production of retinal cells as based on PAX6 expression. Moreover, the presence of RGCs at three weeks post differentiation was supported by HuC/D, TUJ1, and Neurofilament-M (NF-M) immunostaining, as well as by calcium imaging studies that showed a response to glutamate and NMDA. However, about 12\% of the cells had this profile, and more in depth analysis to confirm the RGC identity was not performed. A later study, using a similar approach to differentiate mouse, monkey, and human cells to retinal progenitor cells, produced cells positive for Pax6 and Isl1 as demonstrated by immunostaining. However, the generation of RGCs was not demonstrated in their human culture system\textsuperscript{59}.

More recent publications have improved the generation of retinal neurons while also decreasing the reliance on exogenous signaling molecules for adoption of the anterior neural fate. These techniques follow the same protocol of EB formation followed by culture in neuronal media and transfer to laminin-coated plates. When EBs are cultured in this way, they form neural rosettes that can be manually lifted and again grown in suspension to form neurospheres. This technique allows for enrichment for neuronal cells in the absence of an antibody or reporter driven selection\textsuperscript{60}. Not all rosettes develop toward a retinal progenitor lineage in this culture, with a large number of cells differentiating toward the early forebrain lineage; however, by manually separating optic
vesicle-appearing neurospheres from the main population, which can be distinguished by their appearance under a light microscope, it is possible to enrich for retinal cells\textsuperscript{61}. These cultures have endogenous expression of Dkk1 and Noggin and over time develop to include most neural retina cell types as well as retinal pigment epithelium. RGCs are born in these cultures from day 20 to 50 as indicated by \textit{BRN3} and \textit{CALRETININ} gene expression, but data further characterizing the presumed RGCs was lacking in this study.

Optic vesicle structures formed by Meyer et al.\textsuperscript{60,61} represent an important advancement for recapitulating \textit{in vivo} retinal development, but perhaps even more striking, optic cup-like structures can now be generated from mouse and human ESCs\textsuperscript{24,25}. In this method, ESCs are aggregated into an EB-like floating structure. Over a course of a week, epithelial vesicles evaginate from this sphere and later invaginate to form a two-walled cup-like morphology with retinal progenitor gene expression. This \textit{in vitro} formed optic cup contains a neural retina that is organized like its \textit{in vivo} counterpart. Moreover, these “retinas” express markers of all the appropriate cell types, including RGCs (Pax6, Brn3, Calretinin). The human optic cup takes a longer time to form than its mouse counterpart, and RGC-like cells take about a month to emerge. Although as in previous studies, the RGCs in these cultures were not prioritized for characterization, with expression of only a few RGC-enriched genes providing the supporting evidence, the described retinal tissue structure produced by this method has the same retinal organization as the one seen in normal development, and in this context, provides further support for the observed cells being more likely to be “real” RGCs. Unfortunately, however, like in a real retina, RGCs do not make up a large portion of the cells. In addition to differentiation yield, obtaining RGCs from these structures would
require extracting and purifying the desired cells from the rest of the retinal cells, and efficient methods for achieving this goal have not been reported until the novel work detailed in the following chapters. Despite these limitations, these *in vitro* generated human optic cups represent an invaluable resource for development studies as well as disease modeling. Furthermore, with improved RGC yield and successful RGC isolation, this approach holds promise to be used for RGC generation for drug screening and cell replacement therapies. Similar experiments with iPSCs present the possibility of using patient-derived optic cups for studies of developmental disorders as well as for personalized medicine. It is also interesting to note that unlike in the mouse studies, Notch inhibition by use of DAPT did not appear to increase the number of BRN3 positive cells in the generated human optic cups, although it did increase the number of Cone-Rod Homeobox (CRX) positive cells, suggesting species differences or suboptimal stem cell culture conditions.

1.5 RGC transplantation hurdles and possibilities

Glaucoma generally presents itself as a slow, painless loss of vision starting in the periphery. Many patients will unconsciously augment their behavior to compensate for losses of peripheral vision by turning their heads, thereby allowing the disease to progress for years before treatment is sought. Additionally, retinal damage and cell death often precede detectable visual field loss, further preventing early detection and treatment\(^{62,63}\). As stated in 1.1, glaucoma induced vision loss is permanent and modern medicine does not have a way to restore vision for patients who have become blind, but transplantation of RGCs has been hypothesized to be a potential avenue worthy of scientific exploration.
While the transplantation of RGCs for visual recovery represents a highly challenging problem in biology and medicine, the transplantation of other retinal cell types as cell therapy has provided hope for the field. The transplantation of retinal pigment epithelium (RPE) cells has been shown to be beneficial in animal models of age-related macular degeneration and has even progressed to human trials. Albeit still in the early stages, RPE transplantation has shown no adverse side effects and additional trials aimed at improvement of vision or stall of the disease are underway. The transplantation of retinal neurons is a more difficult task than RPE, but since photoreceptors form far fewer synapses than RGCs and do not possess long axons that must target specific brain areas, photoreceptor transplantation has been a more attractive area for therapy. Photoreceptors transplantation into mice has been demonstrated, with some encouraging improvement of visual function and behavior reported (reviewed in Jayakody et al.). Although visual responses were detected in these studies, it is important to remember that these animals do not regain sight, rather basic visual perception is restored in certain cases.

In order to recover vision following RGC transplantation, it is essential to consider the underlying looming problem of axon guidance in adulthood. During development, growing axons are targeted to proper brain areas via selective guidance cues (reviewed in Haupt and Huber). However, in adulthood, some of these guidance cues are no longer expressed, and the remaining cues may actually prevent re-targeting by de novo axons as these factors were previously utilized to inhibit developing axons from turning back towards the retina (reviewed in Koeberle and Bahr). However, using a combination of genetic alteration and stimulation of signaling pathways, it is possible to
regenerate mouse RGC axons following the optic nerve crush injury model\textsuperscript{79-81}. In these recent reports, RGC axons were able to regenerate all the way to the brain, and some restored visual functions such as optomotor response and circadian photoentrainment were reported\textsuperscript{79}. These results suggest that RGC regeneration is, at least in part, a cell autonomous process and inhibitory guidance cues can be overcome by regenerating RGCs with proper manipulation. Moreover, unlike mammals, some lower vertebrates such as frogs and fish are able to fully and functionally regenerate their optic nerves\textsuperscript{82}, further suggesting that perhaps attractant guidance cues are re-expressed or a cell intrinsic process is ongoing. Additionally, it may be possible to engineer an artificial scaffold to guide RGC axons to proper brain targets\textsuperscript{83,84}. Through combinatorial approaches of bioengineering guidance scaffolds and genetically engineering pluripotent stem cells followed by their subsequent differentiation to RGCs, it may be possible to direct stem cell-derived RGCs to integrate into the retina, repopulate the optic nerve with new axons, and then synapse with the brain’s visual centers. Although no studies have reported stem cell derived RGC axon growth into the optic nerve thus far, a similar experiment has been reported for mouse embryo derived RGC transplantation into adult mice with some donor axonal RGC contribution\textsuperscript{85}.

In addition to biological intervention for retinal regeneration, artificial prosthetic systems for inducing electrical stimulation of the retina have also been developed, which rely on stimulating retinal circuitry downstream of photoreceptors\textsuperscript{86,87}. Since visual perception is a complex process that it is not yet understood well enough on an electrical scale, these artificial systems can only provide rudimentary perception of light rather than true vision, but there is hope that this technology will continue to evolve. Such prosthetic
systems are more difficult to implement in a context of RGC loss, however, yet deep brain stimulation has been proposed as a possible means of bypassing the optic nerve altogether for visual perception recovery\textsuperscript{88}.

Although it is definitely a daunting task, RGC transplantation may prove to be more effective than prosthetic technologies since direct brain stimulation represents a risky procedure and it still remains to be seen whether a computer will be able to recapitulate retinal function and properly communicate with the brain for visual processing. RGC transplantation carries its own risks to be sure, as inappropriate circuitry rewiring of the brain’s visual centers could result in disturbance of brain function or pain, but RGCs themselves will unlikely lead to tumor growth due to their postmitotic nature, and in the event of unfortunate side effects, the newly formed optic nerves could be surgically severed to protect the brain from harm. Regardless of strategy, patients suffering from blindness due to RGC loss have few hopes for recovery besides biological or mechanical RGC replacement. As such, it remains an important goal to conceive a way to replace lost neurons as well as to integrate them into the working visual system. Such experiments will need to first be performed in animal models. Transgenic mice used in optic nerve regeneration experiments should also be useful in this transplantation paradigm, as perhaps regenerating axons will stimulate axon outgrowth of transplanted RGCs. Cross species transplantation of human RGCs into such mouse models or even zebrafish could elucidate cell intrinsic as well as environmental factors that may play a role in visual system recovery. Additionally, due to the likely hostile environment of a degenerating retina, it may be necessary to combine transplantation with neuroprotective\textsuperscript{3} and anti-inflammatory (reviewed in Soto and Howell\textsuperscript{89}) strategies to promote survival and
quell inflammation. Recently, human stem cell derived dopaminergic neurons have been successfully used in treatment of mouse models of Parkinson’s disease. Such triumphs along with the progress of RPE and photoreceptor transplantation fields lay encouraging groundwork to remain optimistic about RGC transplantation as well.

In summary, stem cells remain a potentially life changing tool for many areas of research, including the study of RGC biology and the treatment of optic nerve disease. For glaucoma, stem cells may provide a means to supply NTFs to improve RGC survival, as well as a source of RGCs to be used for neuroprotective drug discovery and for cell replacement therapy. Although RGC differentiation from ESCs and iPSCs is clearly possible, more characterization and confirmation must be carried out in order to sufficiently establish a cellular population as one of RGC lineage. This characterization needs to be based on physical properties such as morphology and electrophysiological responses, as well as gene expression patterns that mimic primary RGCs. Since outside the retina RGCs are in many ways not unique, it is important to array multiple parameters when assessing RGC status and purity. These defined parameters should then be used to compare differentiation protocols in order to improve cell culture methods to raise yield and increase efficiency. Quantitative PCR analysis as well as immunostaining for multiple RGC markers is indispensable, but RNA sequencing analysis between derived cells and their embryonic counterparts is also desired. Additionally, analysis of the DNA methylation patterns of RGC-enriched genes and their specific promoters will provide valuable information. Transplantation analysis to assess the ability of in vitro generated RGCs to integrate into the ganglion cell layer of mouse or rat retinas will also be important. These and related studies will be essential to perform over the coming years
because *in vitro* generation of human RGCs holds tremendous promise for elucidating developmental and disease-related signaling pathways, understanding the mechanisms of neuronal cell death in human cells, and developing novel and more effective approaches for treating patients with optic nerve disease.
Chapter 2: Differentiation of human ESCs to retinal ganglion cells using a CRISPR engineered reporter cell line

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2.1 Introduction

Diseases of the optic nerve often lead to progressive and irreversible vision loss. Glaucoma, the most common of the optic neuropathies, is the second leading cause of vision loss and blindness worldwide\(^91,92\). All current treatments for glaucoma are based on pharmacological, laser-based, or surgical approaches for lowering the eye’s intraocular pressure (IOP). Although such approaches can be effective, sufficient lowering of IOP is not always possible, and RGC loss can still progress despite lowered IOP. In order to develop improved treatment strategies for optic nerve disease, efforts are being made to better understand the mechanisms of axonal injury and RGC death, and to develop neuroprotective approaches to promote RGC survival\(^3\).

Many studies of RGC biology and disease mechanisms have utilized rodent model systems, either *in vivo* animal studies or *in vitro* studies of primary cultures of purified mouse or rat RGCs. Although such studies have provided many important insights, rodent RGCs have potential limitations for the understanding and treatment of human disease. Recent developments in the differentiation of human pluripotent stem cells (hPSCs) into retinal neurons allow for the investigation of human retinal disease using human cells as the model system\(^93\). Additionally, these advances may lead to
development of cell-based therapeutic approaches based on hPSC-derived retinal cells. The greatest progress in such studies has been with hPSC-derived retinal pigment epithelium (RPE) and photoreceptor cells. Stem cell-derived photoreceptor cells that respond to light have been reported, and clinical trials that utilize stem cell-derived RPE cell transplantation as a means to treat age-related macular degeneration (AMD) and Stargardt’s retinal degeneration have begun.

Progress in the differentiation of hPSCs into RGCs has not advanced as rapidly as that of RPE and photoreceptors. Although successful RGC generation has been reported, most published studies have shown expression of a relatively small number of RGC-associated genes and limited physiological characterization of the derived cells, and most importantly, these studies have not provided a method to obtain highly purified populations of human RGCs in large numbers. Here, we describe a simple and scalable protocol for differentiation of human embryonic stem cells (hESCs) to RGCs and their subsequent isolation and characterization. Using a CRISPR-Cas9 based genome editing strategy, we inserted an mCherry fluorescent reporter into the endogenous \textit{BRN3B} (\textit{POU4F2}) open reading frame (ORF) to mark differentiated RGCs. This reporter allows for the use of fluorescence-activated cell sorting (FACS) for generating a purified population of RGCs. We have confirmed that the differentiated cells express multiple RGC markers, exhibit morphological characteristics of RGCs at both the light and electron microscopic levels, demonstrate axonal trafficking of mitochondria, fire multiple spontaneous and induced action potentials, and express functional glutamate receptors. The differentiated RGCs also generate long aligned axons in culture when guided by a nanofiber matrix. Moreover, we identified the small molecule Forskolin as a positive
regulator of stem cell differentiation to RGCs. The availability of well-characterized and highly purified human RGCs will provide a useful cell resource for studying human optic nerve biology and disease, a more medically relevant system for drug discovery efforts, and may help in the development of novel cell-based therapies for the optic neuropathies.

2.2 Results

2.2A Development of an optimized protocol for differentiation of RGCs using an engineered hESC reporter cell line

With the goal of obtaining a population of purified human stem cell-derived RGCs, we generated a knock-in fluorescent mCherry reporter line in H7 hESCs using CRISPR-Cas9 genome editing\textsuperscript{100,101}. We chose to modify the \textit{BRN3B} gene locus for our reporter because \textit{BRN3B} is an important and well-characterized transcription factor and RGC marker\textsuperscript{36,37} whose expression begins early in RGC differentiation and continues in adult cells. \textit{BRN3B} is expressed in a large majority of RGCs, is RGC specific in the retina, and is relatively restricted in its expression throughout the rest of the body\textsuperscript{36,37,102}. In order to maintain \textit{BRN3B} expression and avoid creating a fusion protein of \textit{BRN3B}-mCherry that could affect \textit{BRN3B} function, we tethered together the \textit{BRN3B} ORF and the mCherry fluorescent protein gene with a P2A self-cleaving peptide\textsuperscript{103}. Additionally, we added a membrane signal peptide tag (GAP43 palmitoylation sequence) to the N-terminus of mCherry to guide this protein to the cell membrane. In this configuration, both proteins should be produced from one ORF while retaining their respective cellular localization and functional properties, and \textit{BRN3B} should retain its normal expression levels. We designed a gRNA to target the stop codon of \textit{BRN3B} and synthetized a template plasmid for recombination that contained 5’- and 3’- homology arms of the
BRN3B locus and the P2A-mCherry target integration sequence (Fig. 1a). The expression plasmids for the gRNA, Cas9, and the BRN3B-P2A-mCherry template were electroporated together into H7 hESCs. The cells were then passaged using clonal propagation\textsuperscript{104}, and seventy-two colonies were screened for reporter integration by PCR. One clone, named A81-H7, was found to be homozygous for the reporter (Fig. 1b) and free of predicted off-target mutations. This clone demonstrated a normal karyotype, as determined by G-band analysis of metaphase cells (Fig. 1c). All subsequent differentiation experiments were done using the A81-H7 cell line.

Rather than following one of the more established embryoid body-based methods of inducing retinal differentiation\textsuperscript{24,25,59-61,97,98}, we modified the recently published adherent culture photoreceptor differentiation protocol of Boucherie et al. that relies on the default differentiation of hPSCs to the rostral neuronal phenotype in the presence of extracellular matrix (ECM) cues provided by the addition of Matrigel\textsuperscript{105}. To simplify the protocol, we omitted factors that were included to promote photoreceptor differentiation: taurine, retinoic acid, sonic hedgehog (SHH), and fibroblast growth factors 1 and 2 (FGF1 and FGF2). We were particularly careful not to include factors that could potentially inhibit RGC differentiation\textsuperscript{106}. To support our decision to eliminate these factors, we tested a subset of the factors and other supplements that could be beneficial, but observed no positive effect on RGC differentiation (Fig. 2a,b).

To initiate differentiation, A81-H7 hESCs were dissociated to small clumps of cells and plated on 1% Matrigel-coated dishes before being covered with N2B27 differentiation media containing 2% Matrigel (Fig. 1d). We observed formation of columnar neuroepithelial structures similar to those described in Boucherie, et al.\textsuperscript{105} (Fig.
Figure 1. Generation of an RGC reporter stem cell line.

(a) Schematic illustration depicting the reporter design. CRISPR-Cas9 was used to target the stop codon of *BRN3B* in H7 hESCs. A P2A linked membrane targeted mCherry was added to the *BRN3B* ORF by homologous recombination. Following translation, the BRN3B transcription factor protein is localized to the nucleus while mCherry is targeted to the cell membrane. (b) PCR test for homozygosity. Primers spanning the integration region were used to amplify genomic DNA for comparison between the parental H7 line and the isolated A81-H7 clone. A81-H7 DNA produced only one band of expected integration size, indicating homozygosity of the modified locus. (c) G-banded karyotype analysis of A81-H7 cell line. (d) Schematic of RGC differentiation protocol.
Figure 1

a

CRISPR/Cas9

Stop codon

H7

BRN3B

mCherry

A81-H7

BRN3B

P2A

mCherry

translation

nucleus

membrane

b

c

d

mTeSR1

day 0

N2B27

day 25

Passage hESCs and add N2B27 with 2% Matrigel

mCherry visible
Figure 2. Effects of FBS, Taurine, FGF-8, and FGF-A on differentiation to mCherry+ RGCs.

(a,b) Whole-well microscopy images of day 37 differentiated cultures. (a) FGF-A 50ng/mL was added to differentiating cultures from day 10 to 33. (b) Fetal bovine serum (FBS) - 10%, Taurine – 1 mM, FGF8 – 25 ng/mL or a combination of all three treatments were added to differentiating cultures from day 10 to 30. No visible effect on RGC differentiation was detected from these treatments as compared to the negative control.
Figure 3. Differentiation of mCherry+ RGCs.

(a) Phase microscopy of differentiation over time. Cultures become confluent by day 10.
(b) Phase and fluorescence comparison microscopy of differentiation. mCherry fluorescence becomes visible on day 25. More mCherry+ cells form over time and cell bodies become clearly visible. (a) and (b) Scale bars = 100 μm. (c) Microscopy images on day 38 of differentiation. At this point, mCherry+ cells appear as dense clusters from which fasciculated bundles of neurites extend throughout the dish. Scale bar= 500 μm.
The cells filled out the entirety of the dish by day 10 and initial mCherry+ cells were evident by day 25 (Fig. 3b). The fluorescent signal labeled the entire cell body of the emerging cells, including the soma and neurites of the presumptive RGCs. At later time points, more mCherry+ cells appeared and these cells tended to cluster together and to send out fasciculated bundles of neurites throughout the dish (Fig. 3b,c).

2.2B Differentiating A81-H7 cultures mimic normal retinal development and express markers of all retinal lineages and cell types

To characterize the progression of retinal development of the A81-H7 cells during differentiation, we examined the expression of relevant retinal markers by quantitative real-time PCR (qPCR) over a 30-day time course. Upregulation of expression of 4 mammalian eye field transcription factors (PAX6, LHX2, RAX, SIX3) was observed by day 3 (Fig. 4a). By day 5, these genes had reached relative peak expression levels and their expression remained high over the course of differentiation, except for RAX, which decreased back down to day 3 levels by day 25, consistent with prior reports. SIX6, a gene that comes on during optic vesicle formation, was first detectable by day 5. This was closely followed by an increase on day 10 of expression of VSX2 (Fig. 4b), a marker of neural retina formation. There was also a spike in CRX mRNA expression on day 3 that was followed by a return to baseline on day 5; further CRX expression steadily increased over time (Fig. 4b). To test whether these early CRX transcripts resulted in detectable protein, we performed immunocytochemistry (ICC) for CRX on days 3 and 5 of differentiation, and observed the same pattern with high day 3 expression that
Figure 4

Figure 4. Retinal development is recapitulated in differentiating stem cells.

(a-c) Temporal qPCR analysis of differentiation. Gene expression was first normalized to GAPDH and CREBBP, and then normalized to the value of undifferentiated A81-H7 hESCs. Error bars represent standard error of the mean (SEM). (a) Expression of eye field transcription factors. (b) Expression of optic vesicle, neural retina markers, and neuronal markers. (c) Expression of RGC-associated markers.
Figure 5. CRX protein expression early in differentiation.

Immunofluorescence microscopy images of differentiating cultures on days 3 and 5. Cells were fixed and stained for CRX and Hoechst was used to stain nuclei. Scale bar = 100 µm.
decreased on day 5 (Fig. 5). This unusual biphasic pattern of expression may reflect the finding that there is early CRX expression in proliferating retinal cells, with CRX expression only becoming predominantly expressed in photoreceptors at later stages\textsuperscript{111}. Lastly, we observed that post-mitotic neurons began to emerge starting on day 15, as indicated by an increase in expression of the TUBB3 (βIII tubulin) gene. These results show that the eye field is formed early in this protocol, followed by optic vesicle-like differentiation, and then neural retina differentiation, suggesting that the cultures in general follow the pattern of normal retinal development.

Next, we explored the time course of expression of RGC-enriched genes (Fig. 4c). We quantified the expression of the transcription factors ATOH7, BRN3B, SOX4, and ISL1, as well as the mCherry reporter. One of the earlier genes to show a consistent increase in expression was ATOH7. This gene is expressed in progenitors of all retinal cell types\textsuperscript{112}, but it is essential for the RGC cell fate as ATOH7 knockout mice lack most of their RGCs and have diminished optic nerves\textsuperscript{32,34}. Another gene that has recently been implicated in RGC genesis is SOX4\textsuperscript{44}. ATOH7 and SOX4 both increased in expression starting on day 10, prior to the onset of BRN3B expression, which is consistent with previous reports that suggest that ATOH7 and SOX4 function upstream of BRN3B\textsuperscript{32,44}. As expected, since they are transcribed as a common transcript, BRN3B and mCherry showed similar mRNA expression patterns, with expression being noted on day 25 and increasing into day 30. mCherry protein expression, as assessed by fluorescence, was also first detected on day 25. Lastly, we measured ISL1 expression, whose encoded protein is known to interact with BRN3B to regulate RGC gene expression\textsuperscript{113}. ISL1 was strongly expressed by day 25. A more comprehensive analysis of markers representing all major
Figure 6

(a) qPCR profile for presence of retinal cell types on day 40 of differentiation. Expression was normalized to GAPDH and CREBBP, A81-H7 d0 cells (black) were compared to differentiated cultures (red). Error bars represent SEM. Cell type markers: neurons – VGLUT1, MAP2; Müller glia-GFAP, GS; RGCs - BRN3B; amacrine cells - GAD1, SLC6A9; bipolar cells - CABP5, PRKCA; horizontal cells - LHX1, PROX1; photoreceptors - CRX, RHO, RCVRN; RPE - MITF, RPE65. (b) Immunostaining of day 49 cultures shows remnants of retinal organization. CRX+ photoreceptor progenitors in green and mCherry+ RGCs in magenta. CRX+ cells – white arrows - appear to segregate from mCherry+ axons, suggesting division between the outer nuclear layer and the nerve fiber layer. Scale bar= 500 µm.

Figure 6. Retinal cells are born in differentiating stem cell culture and retain spatial organization.

(a) qPCR profile for presence of retinal cell types on day 40 of differentiation. Expression was normalized to GAPDH and CREBBP, A81-H7 d0 cells (black) were compared to differentiated cultures (red). Error bars represent SEM. Cell type markers: neurons – VGLUT1, MAP2; Müller glia-GFAP, GS; RGCs - BRN3B; amacrine cells - GAD1, SLC6A9; bipolar cells - CABP5, PRKCA; horizontal cells - LHX1, PROX1; photoreceptors - CRX, RHO, RCVRN; RPE - MITF, RPE65. (b) Immunostaining of day 49 cultures shows remnants of retinal organization. CRX+ photoreceptor progenitors in green and mCherry+ RGCs in magenta. CRX+ cells – white arrows - appear to segregate from mCherry+ axons, suggesting division between the outer nuclear layer and the nerve fiber layer. Scale bar= 500 µm.
retinal cell types was performed with day 40 differentiated cells (Fig. 6a). Differentiated day 40 cultures showed a high level of VGLUT1 and MAP2 expression, suggesting a relatively mature neuronal phenotype. In addition to showing expression of RGC markers (BRN3B114), day 40 cells expressed markers for Müller glia (GFAP115), amacrine cells (GAD1114), bipolar cells (CABP5114, PRKCA116), horizontal cells (LHX1114, PROX195), photoreceptor cells (CRX, RHO, RCVRN105), and RPE cells (MITF, RPE65104). Expression of glutamine synthetase (GS115) and solute carrier family 6 (SLC6A9117), markers of Müller glia and amacrine cells, respectively, was also detected, but without an increase over undifferentiated stem cells that already expressed these genes at a high level.

To explore whether the mCherry+ cells demonstrated spatial preferential localization with respect to other retinal cell types, we immunostained differentiated cultures for mCherry and CRX, a photoreceptor marker at later stages of differentiation (Fig. 6b). We observed that the somas of mCherry+ cells were interspersed with CRX+ cells, which were largely absent among the outgrowing mCherry+ neurites. This pattern suggests that the cultures may retain some degree of retinal organization with regards to a ganglion cell layer (GCL) and an outer nuclear layer (ONL). However, the CRX+ cells did not form a continuous laminar ONL-like structure as seen in the in vivo retina, as well as in spontaneously differentiated optic cups24,25. Unlike the 3-dimensional optic cup differentiation method24,25, our adherent cell-based culture method does not restrict cell migration within the dish, likely preventing cells from retaining a well-organized laminar retinal structure and promoting neurite outgrowth of RGCs throughout the dish.
2.2C hESC-derived RGCs can be purified by FACS, survive long term, develop neurite networks in culture, and display ultrastructural properties of \textit{in vivo} RGCs.

In order to obtain a purified population of RGC-like cells, we developed a protocol for flow-sorting the mCherry+ cells in a way that would maintain cell viability. Day 35 or older differentiated A81-H7 cultures were enzymatically dissociated to single cells. In order to develop appropriate FACS gates to define mCherry positive cells, we compared these cells to identically differentiated non-reporter H7 hESCs. We observed a contiguous and wide-spectrum of mCherry expression, with low levels of mCherry expression overlapping with the low level of auto-florescence observed from the non-reporter H7 cells (Fig. 7a). Using a gate that excluded greater than 99.5% of the non-reporter cells, 3-5% of the starting A81-H7 differentiated cell population was mCherry+. Repeat flow analysis of sorted cells right after sorting showed that the sorted population was highly enriched for mCherry+ cells, with 77.7% of cells registering above the threshold gate and an additional 20% registering on the edge of fluorescence (Fig. 8a). Examination of the sorted cells by fluorescence microscopy the day after sorting indicated that the mCherry+ population contained more than 90% fluorescent cells while the mCherry- population contained less than 0.5% fluorescent cells (Fig. 7b).

Additionally, ICC performed on the mCherry+ cells with an anti-BRN3B antibody showed that approximately 90% of the cells were BRN3B+ (Fig. 7c). We also found that by increasing the fluorescence intensity level of the gate used for sorting we could increase the percentage of cells above the original fluorescence threshold to 95.6% (Fig. 8b). Taken together, these results demonstrate that FACS can be used to obtain highly
Figure 7

(a) FACS setup. Differentiated non-reporter H7 hESCs were used to set a threshold for mCherry fluorescence from A81-H7 cells. Density plots and histograms are displayed, with the percent of mCherry fluorescent cells indicated below. (b) Microscopy images of mCherry+ sorted cells one-day post FACS. Phase and fluorescence images are shown. Scale bar=100 µm. (c) Fluorescence microscopy images of mCherry+ and mCherry- sorted cells. Cells were fixed and stained for BRN3B one day after sorting. Scale bar=100 µm.
Figure 8

(a) Day 35 differentiated cells were purified by FACS and then analyzed by flow cytometry. Percent red is determined from a threshold set based on fluorescence of non-reporter H7 cells. Sorted cells are 77.7% above the initial sorting threshold, and are 97.76% positive if cells on the edge of fluorescence are included. (b) Cells were sorted for higher fluorescence as based on the gate shown in red, and then analyzed by flow cytometry with 95.58% of cells being above the non-reporter H7 fluorescence threshold.
purified populations of mCherry/BRN3B positive cells, and that the FACS parameters can be adjusted to achieve the desired balance between purity and yield.

The sorted mCherry+ cells developed neurites within hours post-plating and the cells survived for months. In order to better image developing neurite morphology, we transduced the sorted cells with a constitutive expression GFP lentivirus, which highlighted the dense neurite networks that developed between the cultured cells (Fig. 9a). The neurites developed in a pattern similar to what we have observed previously with primary rodent RGCs. We kept sorted cells alive for over a month after isolation and measured soma sizes on day 95 of culture (Fig. 9b). The cells displayed large cell bodies (soma size: 18.1–26.0 µm, average: 21.2 µm, standard deviation: 2.6 µm, n = 20) which were very similar to human RGC sizes reported in vivo\textsuperscript{118}. To establish whether the purified mCherry+ cells expressed markers characteristic of native RGCs, mCherry+ and mCherry- cell populations from sorted 40-day differentiated cultures were analyzed by qPCR. Expression data was normalized relative to A81-H7 undifferentiated stem cells (Fig. 10a). In the mCherry+ cells, there was significant enrichment of expression of all three members of the \textit{BRN3} transcription factor family\textsuperscript{36} as well as \textit{ATOH7}, \textit{PAX6}, and mCherry. Importantly, mCherry+ cells expressed \textit{BRN3B} at more than 11,000- and 500-fold over the undifferentiated stem cells and mCherry- cells, respectively, further supporting the fidelity of the P2A reporter strategy. A number of other RGC markers also showed enrichment in the mCherry+ cells, including \textit{ISL1}, \textit{ISL2}, \textit{ELAVL3} (also known as \textit{HUC}), \textit{NEFH}, \textit{NEFL}, \textit{SNCG}, \textit{NHLH2}, \textit{NRN1}, and \textit{POU6F2}. As expected, the enrichment was highest for genes that are more specific to RGCs, such as \textit{NRN1}, \textit{POU6F2}, and \textit{NHLH2}\textsuperscript{119-121}, compared to \textit{ELAVL3} and \textit{NEFH/L}\textsuperscript{122,123}. Lastly, since \textit{SOX4} and \textit{SOX11}
Figure 9. FACS sorted cells survive long term and develop long neurites.

(a) Microscopy images of FACS-purified mCherry+ cells transduced with lenti-GFP and cultured for 18 days after transduction. GFP fluorescent dense neurite networks are visible. (b) Microscopy images of a long-term culture of FACS-purified mCherry+ cells. Cells were transduced with lenti-GFP after sorting on day 43 of differentiation and cultured for 52 days post-sort. Scale bars=100 µm.
Figure 10. qPCR analysis of sorted cells.

(a,b,c) Day 40 sorted mCherry+/- cells were analyzed for RGC-associated genes. Expression was normalized to GAPDH and CREBBP. Error bars represent SEM. (a) Normalized expression to undifferentiated A81-H7 hESCs shows enrichment of RGC-genes in mCherry+ cells. (b,c) mCherry+/- cells compared to undifferentiated A81-H7 hESCs. (b) All three cell populations express the RGC-enriched markers of THY1 and RBPMS. (c) Only mCherry+ cells express the iPRGC marker OPN4 (melanopsin). Two different primer pairs were used to confirm OPN4 expression.
knockout mice exhibit RGC loss, we looked for differences in expression of these genes in the sorted cells as well. However, the mCherry+ cells exhibited no SOX4 enrichment and very little SOX11 enrichment. Notably, these factors are also expressed in other retinal cell types and may only be RGC specific very early during differentiation. An analogous situation may explain our observations for the RGC markers THY1 and RBPMS. As expected, we observed strong expression of THY1 and RBPMS in the mCherry+ cells (Fig. 10b). However, there was also high expression of these markers in the starting A81-H7 cell population and in the mCherry- cell population, consistent with the finding that expression of these genes outside the eye is not RGC-specific. We also examined expression of OPN4, a marker of intrinsically photosensitive RGCs (ipRGCs). qPCR revealed a low but clearly detectable level of expression of OPN4 (Fig. 10c) in the mCherry+ cells. A low level of expression in a mixed RGC population would be expected since ipRGCs represent only 1-2% of endogenous RGCs.

We also tested the mCherry+ sorted cells for a variety of RGC-markers at the protein level (Fig. 11). ICC showed evidence of expression of the following RGC-associated proteins: pan-BRN3, BRN3B, BRN3A, NEFH, PAX6, THY1, RBPMS, ISL1, and SNCG, as well as the mCherry protein. The more general neuronal markers TUJ1, MAP2, and NEUN, which are expressed in RGCs, were also detected.

Additionally, we tested relative expression of non-RGC markers in the mCherry+ and mCherry- cell populations. In the mCherry- population we detected enrichment of non-RGC retinal markers for amacrine cells, bipolar cells, horizontal cells, and RPE (Fig. 12a). To test for photoreceptor and Müller glia markers we immunostained the cells for
Figure 11. Sorted mCherry+ cells stain for RGC-enriched proteins.

Fluorescence microscopy images of mCherry+ sorted cells one day after sorting (a) or 6 days after sorting (b). mCherry+ cells stained positive for the mCherry protein, the general neuronal markers TUJ1, MAP2, and NEUN, and the more RGC-enriched markers pan-BRN3, BRN3B, BRN3A, NEFH, PAX6, THY1, RBPMS, ISL1, and SNCG. Hoechst was used for DNA staining. Scale bars = 100 μm.
Figure 12

(a) Sorted mCherry+ cells stain negative for CRX and GFAP while some mCherry- cells stain positive. (b) Day 63 sorted mCherry+/− cells were analyzed for expression of retinal cell type associated genes. mCherry+ fraction shown by red bars, mCherry- fraction shown by clear bars. Expression was normalized to *CREBBP*. Error bars represent SEM.
CRX and GFAP, respectively, and did not observe expression of either marker in mCherry+ cells (Fig. 12b).

We also assessed the FACS-sorted mCherry+ cells at the ultrastructural level by transmission electron microscopy (TEM). Consistent with observations of in vivo RGCs, A81-H7 RGC somas demonstrated large euchromatic nuclei, prominent rough endoplasmic reticula, and axon hillocks (Fig. 13a). TEM of neuronal processes ranged in caliber diameter from 0.2 µm to 2 µm (Fig. 13b). While it was not possible to definitively differentiate axons from dendrites by TEM of adherent cultures of sorted RGCs, the neuronal processes contained elongated mitochondria, smooth endoplasmic reticula, and longitudinally-oriented neurofilaments, which are all features observed in RGC axons in the human nerve fiber layer and optic nerve.

2.2D FACS purified mCherry+ cells demonstrate physiological properties associated with mature RGCs

Calcium imaging was performed to assess the electrophysiological behavior of the sorted cells. Using the calcium sensitive dye Fluo-4AM, we observed spontaneous, transient spikes in intracellular calcium concentration in the sorted cells that appeared to be indicative of neuronal electrophysiological activity.

A hallmark of retinal ganglion cells, as compared to other retinal neurons, is their ability to encode membrane depolarizations in trains of action potentials. To determine whether the hESC-derived RGCs demonstrated this important feature, we performed whole-cell patch-clamp recordings. We observed that the RGCs were capable of
Figure 13. Sorted mCherry+ cells show ultrastructural properties consistent with RGCs.

(a) TEM of mCherry+ RGCs one week post-sorting. A81-H7 RGCs feature a large euchromatic nucleus, prominent rough endoplasmic reticula (arrowheads, bolded inset is a magnification of the dashed box), and an axon hillock (arrow). (b) TEM of neuronal processes showed that processes ranged in caliber from 0.2 to 2 µm. This representative micrograph displays processes containing longitudinally arranged neurofilament, mitochondria, and smooth endoplasmic reticula. Scale bars are (a) 2 µm and (b) 500 nm. This figure was generated by Chung-ha Davis from the laboratory of Dr. Nicholas Marsh-Armstrong of Kennedy Krieger Institute and Johns Hopkins School of Medicine.
spontaneous generation of action potentials driven by intrinsic membrane potential depolarizations (Fig. 14a). Furthermore, injecting current through the somatic recording electrode elicited action potentials in all cells studied (Fig. 14b). Nearly all (11/13) cells generated increasing numbers of action potentials in response to progressively larger current injections (Fig. 14c). The two remaining ganglion cells generated one or two action potentials. This distribution of cellular responses is consistent with previous studies in mammalian model systems examining RGC physiology at various stages of development\textsuperscript{131}.

Ganglion cells receive excitatory, glutamatergic inputs from bipolar cells in the intact retina. To test whether cultured RGCs also express glutamate receptors, we utilized local pressure application (“puff”) of the AMPA/kainate receptor agonist kainate. A brief puff of kainate evoked a current that was reversibly blocked by the AMPA/kainate receptor antagonist NBQX (Fig. 14d). We confirmed this result in eight neurons from two separate cultures, confirming that these hESC-derived RGCs mature in culture such that they express the AMPA/kainate receptors necessary for excitatory transmission.

To test whether sorted RGCs display properties of axonal flow, we transfected the cells with an AAV virus mitochondria fluorescent reporter. Imaging of these cultures over time demonstrated mitochondria movement throughout RGC axonic processes.

2.2E Aligned nanofiber scaffolds can guide RGC axonal development

As a step toward modeling the optic nerve \textit{in vitro}, we developed a system to guide RGC axonal outgrowth. Although hESC-derived RGCs demonstrate relatively long
Figure 14. Sorted mCherry+ cells show electrophysiological properties consistent with RGCs.

(a) Spontaneous action potentials recorded in a cultured RGC under whole-cell current clamp. Time scale applies to all panels. (b) Injection of depolarizing current (bottom) causes the cell to fire a train of action potentials throughout the current injection (top), same cell as in (a). (c) A second representative example cell showing 20, 40, 60, or 80 pA depolarizing current injections (bottom: left to right) and the corresponding increases in the number of action potentials generated (top). Vm is membrane voltage. (d) Responses to 50 ms local dendritic puff applications of 100 μM kainate in a cultured RGC under whole-cell voltage-clamp (Vm = -70mV). Left: average of baseline responses. Center: average of responses after bath application of AMPA/KA receptor antagonist NBQX (20 mM). Right: average of responses after NBQX washout. This figure was generated by Dr. Justin Kerr from the laboratory of Dr. Jeffrey S. Diamond of the National Institutes of Health.
neurite-like processes in culture (Fig. 3c), these processes generally appear disorganized, presumably due to the lack of appropriate extracellular matrix and target-derived axonal guidance cues. With these randomly arranged axon bundles, it would be difficult to measure axonal extension, injury response, and myelination behavior of the axons. Since aligned nanofiber scaffolds have been shown to effectively guide axonal outgrowth in vitro, we tested whether RGC neurites could be guided to form long axons on polymer nanofibers that would better resemble the highly aligned nature of the endogenous optic nerve. In this study, we chose poly-ε-caprolactone (PCL) to prepare nanofibers due to its strong biocompatibility track record and the ease of processing for electrospinning.

Aligned PCL fibers with an average diameter of 630 nm were selected based on a pilot experiment showing that fibers with diameters in the range of 500 nm to 1.5 µm mediated the most effective directional migration for neurons (data not shown). These aligned fibers were surface coated with Matrigel (100 µg/ml) to improve their adhesion property to RGCs. We dissociated differentiated retinal cultures using brief enzymatic treatment to generate cell clusters and then encapsulated these clusters in Matrigel droplets plated on aligned PCL nanofibers (Fig. 15a). Robust fluorescent neurite extension, originating from the cell clusters, was observed along the nanofibers, and this outgrowth could be monitored over time using live cell imaging. It was clear from video microscopy that axons were sampling and extending on the tracks of the aligned fiber substrate. To determine whether the long projections we observed were axonal, we immunostained the cultures for mCherry, TAU, and MAP2. The long projections were positive for mCherry and TAU, indicating that they originated from BRN3B+/RGC cells in the clusters (Fig. 15b). We then applied TAU/MAP2 staining to determine whether
Figure 15. Differentiated cultures grow aligned axons on nanofiber scaffolds.

(a) Fluorescence microscopy image for mCherry. Differentiated retinal cultures were dissociated and re-plated in Matrigel droplets on an aligned nanofiber scaffold that guides emerging neurite outgrowth. Scale bar = 1 mm. (b,c) Differentiated day 49 cultures were dissociated and plated on nanofibers in Matrigel droplets for 18 days before fixation. (b) Immunostaining for TAU and mCherry proteins. Hoechst staining shows that while cells were largely restricted in their migration, the cultures sprouted long projections that stained positive for TAU, suggesting axonal identity, and mCherry, suggesting RGC origin. Scale bar= 500 µm. (c) Immunostaining for TAU and MAP2 suggests that the RGC emanating projections are largely axonal since MAP2 appears to be restricted to the soma containing area of the dish (indicated by white arrows). Scale bar= 500 µm. (d) Matrigel droplets were loaded into a limited diffusion chamber of a 5 mm length. With limited diffusion, signaling cues from neighboring clusters may accelerate neurite outgrowth.
these two proteins had localized separately in the culture, an indication of axonal versus dendritic compartmentalization. Indeed, the long projections stained positive for TAU, but negative for MAP2, confirming that they were axons. In contrast, consistent with the pattern seen in differentiated neurons, MAP2+ dendrites were localized closer to the Matrigel encapsulated cell somas (Fig. 15c).

We also designed a limited diffusion chamber to enhance axon outgrowth in the differentiated RGC culture and to move closer to the development of an in vitro optic nerve model (Fig. 15d). By placing two Matrigel encapsulated cell clusters in two microwells connected by a microchannel on top of an aligned nanofiber sheet, we observed robust axonal outgrowth traversing the entire length of the chamber (5 mm) in 10 days, indicating an average axonal growth rate of about 0.5 mm/day. Interestingly, although the axonal growth rate is not known for human primary RGCs, the rate of axonal growth in embryonic day 15 mice has been reported to be about 1 mm/day, a rate similar to the one that we observed under these in vitro culture conditions with hESC-derived RGCs. These data demonstrate that these hESC-derived RGCs can extend their axons in an organized fashion when guided by an artificial matrix.

2.2F Addition of Forskolin can improve stem cell differentiation to RGCs

Although the ability to purify BRN3B+ cells through the use of the mCherry reporter makes the efficiency of differentiation to RGCs less of an issue since more cells could be sorted overall, it would still be desirable to improve the efficiency of RGC differentiation beyond the 3-5% that we obtain with our first protocol. We therefore
screened a number of small molecules in an attempt to increase the percentage of cells that differentiate to RGCs. Most of the tested molecules were ineffective, toxic, or inhibitory to RGC differentiation (Fig. 2). However, we found that Forskolin, an activator of adenylate cyclase that is known to increase RGC neurite outgrowth\textsuperscript{138} and survival\textsuperscript{17,139} as well as to augment differentiation in other systems\textsuperscript{140,141}, had a dramatic positive effect on differentiation when added starting on day 1 (Fig. 16a). The effect of Forskolin on RGC lineage commitment and differentiation appears to be mediated early, as exposure to Forskolin for only the first week of culture had an effect comparable in magnitude to that observed when it was present continuously from day 1 to 30 (Fig. 16b). Moreover, the addition of Forskolin starting on day 5 or later had no positive effect on RGC differentiation (data not shown). Since differentiation efficiency in Figure 16b was unusually low in the control, we repeated Forskolin experiments additional times to validate its positive effect, and observed consistent RGC differentiation promoting activity (Fig. 16c). We performed a dose-response experiment to establish an optimal Forskolin dose, but saw limited effect from increasing the concentration from 5 to 50 µM. We also assessed the effect of Forskolin on RGC marker expression. As measured by qPCR, sorted mCherry+ cells differentiated with the Forskolin treatment protocol demonstrated enrichment for \textit{BRN3A}, \textit{BRN3B}, \textit{BRN3C}, \textit{PAX6}, \textit{ISL1}, \textit{ISL2}, \textit{NHLH2}, and \textit{POU6F2} expression that was similar to enrichment observed in mCherry+ cells generated in the absence of Forskolin (Fig. 17). Additionally, we checked the neurochemical behavior of Forskolin-treated sorted cells by calcium imaging, and observed calcium transients similar to those observed in cells differentiated without Forskolin. Lastly, in attempt to gain insight into the mechanism by which
Figure 16. Addition of Forskolin improves stem cell differentiation to RGCs.

(a) Whole-well microscopy scans of day 40 differentiated cultures. Addition of 25 μM Forskolin from day 1 of differentiation until day 30 increases the number of differentiating mCherry+ cells as compared to control. (b) Flow cytometry analysis for percent of mCherry+ cells. Day 40 differentiated cells were treated with Forskolin or DMSO for different times. Addition of 25 μM Forskolin from day 1 of differentiation to day 6, 10, 20, or 30 increased the percentage of mCherry+ cells to similar extents, suggesting that the critical period for its mechanism of action is in the first week. P values were 0.0005, 0.0032, 0.049, and 0.005, respectively. (c) Flow cytometry analysis for percent of mCherry+ cells in differentiated cultures at day 40 and day 35. Cells were treated with 25 μM Forskolin or DMSO from days 1 to 6 of differentiation. P values were 0.04 and 0.01, respectively. *p<0.01, **p<0.05. N=3. Unpaired two-tailed t-test was used to compare Forskolin treated samples with control. Error bars represent standard deviation.
Figure 17

qPCR analysis of Forskolin treated differentiated day 40 cells. Forskolin was applied from day 1 to 6 of differentiation. Gene expression was normalized to GAPDH and CREBBP. mCherry+ cells were normalized to mCherry- cells. Error bars represent SEM. mCherry+ cells show enrichment for expression of the RGC-associated genes BRN3A, BRN3B, BRN3C, ISL1, ISL2, NHLH2, POU6F2, and PAX6.
Figure 18. Analysis of Forskolin induced gene expression changes during differentiation.

Differentiating cultures were treated with either DMSO or 25 µM Forskolin for 10 days and RNA was extracted and analyzed on days 5 and 10. Expression was normalized to GAPDH and CREBBP. Error bars represent SEM. CFX Manager software was used to perform a two-tailed t-test to calculate the p-values. **p<0.05, *p<0.01. N=3. P values for day 5 were: SIX3 - 0.000056, LHX2 - 0.010169, PAX6 - 0.021039, RAX - 0.000021, and ATOH7 - 0.002231. P values for day 10 were: SIX3 - 0.000029, LHX2 - 0.000007, RAX p< 0.000000, SIX6 - 0.000195, VSX2 - 0.013346, ATOH7 - 0.032986, and SOX4 - 0.001031.
Forskolin promotes RGC differentiation, we assessed gene expression changes by qPCR (Fig. 18). Perhaps surprisingly, Forskolin upregulated expression of the eye field transcription factors on day 5 of differentiation, but on day 10, Forskolin decreased their expression and increased expression of SIX6 and ATOH7.

2.3 Discussion

We have developed a simple and scalable system for differentiation and purification of human RGCs from hPSCs using a genetically engineered RGC reporter hESC line. To engineer the RGC reporter line, we utilized CRISPR-Cas9 technology to knock in a P2A-mCherry sequence into the 3’ end of the BRN3B ORF, an RGC marker gene. Then to derive RGCs we modified a recently published protocol designed for generation of photoreceptors\textsuperscript{105}. This protocol appealed to us as a starting point because it does not require embryoid body formation, neural rosette picking, or tedious suspension cultures\textsuperscript{24,25,59,60,95,99}, and yet it delivers efficient retinal differentiation. Since the protocol is an adherent culture protocol, it can easily be performed in 96 well plates for efficient screening or in tissue culture flasks of expanding sizes to generate larger numbers of cells. To increase RGC yield, we removed many additives that are unnecessary for RGCs, and which may actually inhibit RGC differentiation. Using our modified protocol and the engineered cell line, we first observed fluorescent cells appearing by day 25 of differentiation, which coincided with the timing of detectable BRN3B and mCherry mRNA expression. We then went on to characterize the RGC differentiation process over time. Our qPCR data suggests that the cultures followed the normal retinal development paradigm as has been described in other species: markers of the prospective eye field
increased in expression early in the differentiation process, followed by markers of the “neural retina,” and finally markers of differentiated RGCs. Furthermore, on day 40 of differentiation, we detected markers of all retinal cell types by qPCR. In addition to retinal gene expression, we also found that the mCherry+ cells were localized in proximity to CRX+ cells in our cultures, suggesting that not only were the mCherry+/BRN3B+ cells likely to be of retinal origin themselves, i.e. RGCs, but also suggesting that the differentiating retinas had retained some measure of retinal organization despite the cultures having no rigid structure.

Building on these promising results, we developed a protocol to FACS purify the fluorescent cells. The sorted cells were characterized by qPCR and ICC for a wide range of RGC-enriched genes to confirm their cell identity. We found the sorted mCherry+ cells to be consistent with the RGC phenotype as they expressed all of the RGC markers we analyzed. However, few RGC markers, if any, are completely restricted to RGCs when compared to the nervous system and the rest of the body as a whole. For instance, although the BRN3 transcription factors are restricted to RGCs in the retina, they are also expressed in the auditory system and the somatosensory system\textsuperscript{102}. We confirmed that the sorted cells expressed BRN3C in addition to BRN3B. These two factors do not overlap in their spatial expression in the auditory system\textsuperscript{102}, thus indicating that the sorted cells were not of auditory origin. Then to distinguish somatosensory neurons from our sorted cells, we profiled the mCherry+ cells for PAX6 by ICC and qPCR. PAX6 is required for the development of the eye and the brain. It is expressed in all retinal progenitor cells but is restricted to RGCs and amacrine cells later in development\textsuperscript{142}. While somatosensory neurons are not known to express PAX6, according to the BioGPS database\textsuperscript{143-145}, our
cells expressed PAX6 at a high level. Additionally, we noticed that our cells expressed *ISL1* more than *ISL2*, a pattern that also appears in retinal cells but not in the somatosensory system. Moreover, the mCherry+ cells expressed *RBPMS*, a gene that has recently been suggested to be RGC-specific in the nervous system. Based upon these results, we conclude that our mCherry+ cells are, indeed, of the RGC cell fate. Importantly, we also detected low-level expression of melanopsin (OPN4), a pigment protein expressed in a small subset of RGCs, i.e. ipRGCs. It will be interesting to assess what other subtypes of RGCs can be detected in these cultures, in order to establish whether certain subtypes are preferentially produced and if these preferences can be altered with small molecules or by transcriptional modulation.

In addition to expressing markers consistent with the RGC phenotype, mCherry+ cells also demonstrated ultrastructural properties consistent with *in vivo* RGCs as assessed by TEM, their mitochondria exhibited flow throughout the cells’ axons, and the cells appeared to be functional at the electrophysiological level. Using calcium imaging, we were able to detect calcium transients typical of neuronal electrical activity. Then, using whole-cell patch-clamp, we showed that the sorted cells fired multiple action potentials, spontaneously and when exposed to current. Moreover, the cells demonstrated glutamate receptors as indicated by their ability to respond to the AMPA receptor agonist kainate, as expected for native RGCs. Together, this data suggests that these cells have the means to receive input and transmit output.

In an effort to build an *in vitro* optic nerve-like model and to provide a means to study RGC axonal outgrowth, we integrated our stem cell cultures with biomaterials to achieve axonal guidance. When differentiated retinal cultures were seeded onto nanofiber
scaffolds, the cells responded to these scaffolds with guided axons growing for several millimeters in length in a time span of just two weeks. Due to their aligned nature, such scaffolds can be used to further probe RGC axonal guidance and through live imaging of the mCherry+ cells, axonal growth rates can be monitored in real time. Furthermore, this model may be used to better assess axonal repair following axonal injury in culture. It should therefore be possible to develop culture-based models of optic nerve injury and regeneration to partially mimic human disease. If RGCs are to be used for cell-based therapy in the future, engineered nanofibers may also provide a viable technology to guide the RGC axons to their proper targets in the brain. Additionally, it may be possible to enhance the activity of biomaterial scaffolds through conjugation of guidance molecules directly to the scaffolds, thereby combining physical and chemical guidance to increase and direct axonal growth.

One of the advantages of incorporating a reporter into human stem cell-based differentiation paradigms is the ability to use reporter fluorescence as a readout in screens for differentiation modulating compounds. Through a pilot small molecule screen, we found that Forskolin, when added early in differentiation, results in an increased percentage of RGCs. Forskolin is an activator of adenylate cyclase, which increases intracellular concentrations of cAMP. This molecule has been previously reported to improve RGC survival and promote neurite outgrowth, and to aid in the direct reprogramming of fibroblasts to iPSCs and cholinergic neurons. To explore Forskolin’s activity on differentiating hESCs, we examined its effect on expression of selected retinal transcription factors. Notably, while neurogenesis did not appear to be affected as based on NCAM1 expression, Forskolin did increase expression of the eye
field transcription factors on day 5, suggesting a pro-retinal effect. However, in the presence of Forskolin on day 10, these genes had lower expression while SIX6 and ATOH7 were increased, perhaps suggesting an effect on optic vesicle or RGC formation. While the mechanism of Forskolin’s RGC differentiation promoting activity requires further study, its addition to the protocol increases RGC generation and makes possible the production of 2.5 million RGCs per 24 well tissue culture plate. This brings the concept of using sorted human RGCs for large-scale drug screening closer to a practical reality. As large numbers of cells would be required for high throughput drug screening for studies of RGC survival, and for potential toxicology screens of compounds prior to clinical trials, we have begun a larger screen to identify additional small molecules that promote RGC differentiation. Identification of such molecules would not only allow for increased RGC yield, but would also provide molecular probes that could help further define RGC differentiation and biology.

The use of CRISPR-based gene editing approaches to make reporter lines, such as the BRN3B reporter described here, can also be used for the introduction of human mutations associated with optic nerve disease. An obvious early application of such technology will be the generation of human RGCs from ESCs and/or iPSCs carrying the E50K optineurin mutation in combination with the BRN3B reporter. E50K glaucoma patients appear to be particularly sensitive to RGC injury and death\(^\text{146}\). Development of a human RGC cell culture system that mimics E50K biology \textit{in vitro} would allow the direct comparison of pertinent mutant versus wild-type cells and thereby aid greatly in efforts to elucidate novel disease mechanisms and develop potential therapeutics.
2.4 Methods

2.4A Plasmid construction

The CRISPR guide RNA (gRNA) and donor template plasmids used for the BRN3B-P2A-mCherry reporter line generation were constructed as previously described\textsuperscript{101}. To generate the mCherry-targeting vector, we first synthesized a gBlock (IDT) encoding the P2A sequence, a GAP43 palmitoylation sequence, and mCherry. This full sequence was inserted into the pUC19 vector (NEB) by Gibson Assembly (NEB). The left and right \textit{BRN3B} homology arms, (898 base pairs) and (1,120 base pairs) respectively, were amplified from human genomic DNA using the following primers:

left forward: CGCCGAGGCTCTGCGAGCCG

left reverse: AATGCCGGCGGAATATTTCATTCTTTTC

right forward: TAGAAGACTCTTGGCCTCTCCAGAG

right reverse: TGCATCGGTCATGCTTCCAACTGC

These homology arms were inserted into the targeting vector using Gibson Assembly and the final donor vector was verified by sequencing prior to genome targeting. The targeting gRNA sequence (GCCAAGAGTCTTCTAAATGCCGG) was cloned into a U6-driven gRNA expression vector (Addgene \#41824) as described\textsuperscript{147}.

2.4B Reporter line generation

Gene editing of H7 hESCs (WiCell) was performed as previously described with slight modifications\textsuperscript{101}. Electroporation was performed using the Neon Transfection
System 10 µL Kit (Invitrogen) according to the manufacturer’s instructions. Briefly, semi-confluent H7 hESC cells were cultured in mTeSR1 media (Stemcell Technologies) containing 10 µM Rho Kinase inhibitor DDD00033325 (EMD Millipore) 24 hours before passaging. H7 colonies were digested with Accutase (Sigma) for 5 minutes to form a single cell suspension. The cell suspension was centrifuged, and following supernatant removal, the cell pellet was left on ice for 15 minutes before resuspension in R-buffer containing three separate plasmids encoding the gRNA, Cas9 (Addgene #41815)\(^1\)\(^4\)\(^7\), and the donor template. The following electroporation parameters were used: voltage, 1,400 V; interval, 30 ms; 1 pulse. After electroporation, the cell suspension was slowly transferred to mTeSR1 medium containing 10 µM DDD00033325 and incubated at room temperature for 20 minutes before plating onto Matrigel (BD Biosciences) coated dishes. Cells were cultured until they reached confluency suitable for passaging, at which time they were split as a single cell suspension and grown at a low density of 500 cells per well of a 6-well plate. Once colonies formed, 72 individual colonies were manually picked and screened for reporter integration by PCR using the following forward and reverse primers (5’-3’):

forward: GGAGAAGCTGGACCTGAAGAAAAACGTGGTG

reverse: CCTTGGTGAAATCTAAAATCTGAAGGGCAAACACC

These primers amplify the genomic region containing the integration site, therefore showing if one or both alleles contain the reporter sequence. A homozygous reporter integration cell line was identified. This line, named A81-H7, was further validated by DNA sequencing and its karyotype was analyzed using standard procedures at Cell Line Genetics. To test this line for CRISPR off-target effects, we sequenced the 3 most likely...
off-targets and 2 additional predicted exonic off-targets as assessed by the CCTop online tool (Table 2)\textsuperscript{148}. All predicted sites were free of mutations.

2.4C Human ESC maintenance

H7 and A81-H7 cells were maintained by clonal propagation in mTeSR1 media (Stemcell Technologies) on growth factor-reduced Matrigel coated plates\textsuperscript{104} at 10% CO\textsubscript{2}/5% O\textsubscript{2}. hESC colonies were passaged by dissociation with Accutase (Sigma) or TrypLE Express (Life Technologies) for 5 minutes, followed by ten-fold dilution of the cell suspension with DMEM/F12 (Life Technologies). After centrifugation at 150xg for 6 minutes, the cell pellet was resuspended in mTeSR1 media containing 5 µM blebbistatin (Sigma). The cells were cultured at a density of 15,000 cells per well of a 6 well tissue culture plate. Two days after passage, the medium was replaced with mTeSR1, which was then changed daily.

2.4D Human ESC differentiation towards RGC lineage

hESCs differentiation was induced as described previously\textsuperscript{105}. Briefly, hESCs were incubated with TrypLE Express (Life Technologies) for 1 minute. Using a cell scraper (Sarstedt), colonies were lifted off the plate, transferred into DMEM/F12, and centrifuged at 150xg for 6 minutes. The cell pellet was gently dissociated to a clump suspension via gentle pipetting in N2B27 differentiation media [1:1 mix of DMEM/F12 and Neurobasal (Life Technologies) with 1× GlutaMAX Supplement (Life Technologies), 1× antibiotic-antimycotic (Invitrogen), 1% N2 Supplement (Life Technologies), and 2% B27 Supplement (Life Technologies)]. The cell clump suspension
was then distributed to a 24 well tissue culture plate (Falcon) pre-coated with 1% Matrigel. One well of a 6-well hESC culture was used per 24 well differentiation plate. After one hour at 37°C in 5% CO₂/20% O₂, enough time for cell clumps to attach, media was replaced with cold (4°C) N2B27 containing 2% Matrigel. Media was then changed every other day using N2B27 without Matrigel. The day of cell dissociation and 2% Matrigel addition is referred to as day 0 of RGC differentiation. For some experiments, 25 µM Forskolin (Stemcell Technologies) was included in the media from differentiation day 1 to day 30 (unless otherwise specified). Other compounds we tested starting on day 10 were 25 ng/mL FGF-8 (PeproTech), 50 ng/mL FGF-A (PeproTech), 1 mM Taurine (Sigma), or 10% fetal bovine serum (Life Technologies).

2.4E Fluorescence-activated cell sorting

To set the sorting gates, we used identically differentiated reporter-less H7 hESCs to define the mCherry+ cell population. Although H7 control cells exhibited low auto-fluorescent signal within our target gated population, we chose not to change this gate stringency since it would decrease yield. Side scatter height versus width linear alignment filters were used during sorting to minimize cell aggregates.

To prepare cells for sorting, differentiated cultures were dissociated into a single cell suspension. Differentiated cultures (day 35-63) were washed with phosphate buffered saline (PBS, pH 7.4) and then incubated with TrypLE Express for 15 minutes at 37°C. The TrypLE was replaced with Accumax (Sigma) and incubated an additional 45 minutes. Cells were then gently triturated using a P1000 pipette, and DMEM/F12 was
added to the cell suspension and centrifuged at 150xg for 6 minutes. The cell pellet was resuspended in Live Cell Imaging Solution (Life Technologies) and further triturated. The single cell suspension was then passed through a cell strainer (BD Falcon) prior to analysis and sorting with an SH-800 Cell Sorter (Sony). Following sorting, the resulting cell suspension was centrifuged at 150xg for 6 minutes. The cells were then resuspended in growth medium N2B27 with 10 μM Forskolin and 10 ng/mL CNTF (PeproTech) and plated on Matrigel pre-coated plates. To prevent microbial contamination, 2 μg/mL doxycycline (Sigma) and/or 1 μg/mL mitomycin-C (Sigma) were added for 1 to 2 days of culture post sorting.

2.4F Electrophysiology

To prepare cells for electrophysiology, FACS sorted cells from day 50 of differentiation were seeded on Matrigel coated glass coverslips (Fisher Scientific) inserted into culture plates at a density of 50,000-100,000 cells/cm². Cells were fed with N2B27 media containing 2 μg/mL doxycycline, 1 μg/mL mitomycin-C, 10 μM Forskolin, and 10 ng/mL CNTF. After 24 hours, the media was replaced with N2B27 supplemented with Forskolin, CNTF, and doxycycline but without mitomycin-C. At 72 hours after plating, cell media was replaced with cold (4°C) N2B27 media containing 1% Matrigel in order to prevent cells from detaching from the glass coverslip. The next day, media was replaced with N2B27 containing 10 μM Forskolin, 10 ng/mL CNTF, 50 ng/mL BDNF (PeproTech), 10 ng/mL GDNF (PeproTech), and 10 ng/mL NT4 (PeproTech) and cultured in this media for 19-21 days until electrophysiology recording.
On day of recording, small portions of the coverslip were transferred to a microscope for electrophysiology experiments. Cells were superfused with Ames medium equilibrated with 95% O₂/5% CO₂, warmed to ~35°C. Patch electrodes had tip resistances of 4–6 MΩ when filled with internal patch solution containing (in mM): 100 potassium methanesulfonate, 5 KCl, 5 NaCl, 10 HEPES, 4 EGTA, 10 phosphocreatine (Na salt), 4 Mg-ATP, and 0.4 Na-GTP. Access resistance was low (~10-15 MΩ) and was not compensated. Current-clamp recordings (Axopatch 1D amplifier, Axon Instruments) were acquired via a 16-bit Analog-digital interface (Instrutech Corp.; 10 kHz sample rate) and a low-pass filter (5 kHz). Data acquisition and analysis was controlled with custom macros written in Igor Pro (Wavemetrics). Extracellular recordings were collected prior to establishing the whole-cell configuration. For whole-cell current-clamp recordings, individual trials lasted 3-6 seconds and included 1-3.5 second step current injections generated by the patch-clamp amplifier. Successive trials varied the injected current from 20-140 pA, in 20 pA increments, to depolarize the membrane potential and to test for action potential responses. Some cells had a tendency to spontaneously depolarize and generate trains of action potentials at rest. For these cells, a small constant current was applied to hyperpolarize the membrane potential near -60 mV, beneath its spike threshold, thereby providing a clear baseline for subsequent action potential responses.

For kainate responses, whole-cell voltage-clamp recording were used, approximately 10 individual trials were averaged for a cell or particular response condition. Kainate (100 μM; Tocris) was dissolved in HEPES-buffered Ames and loaded into patch electrodes connected to a Picospritzer (Parker Instrumentation). The
Picospritzer was triggered by the acquisition software to deliver 50-100 ms puffs of kainate (0.5 bars pressure) to proximal dendrites of recorded cells.

2.4G Lentivirus production and transduction of sorted cells

Lentivirus was produced using calcium phosphate transfection as described\textsuperscript{149}. One day before transfection 5 × 10\textsuperscript{6} 293T cells (Clontech) were seeded onto a 10-cm cell culture plate. The following day cells were transfected with 15 µg MD2.G (Addgene #12259), 6 µg MDL/pRRE (Addgene #12251), 6 µg RSV/rev (Addgene #12253), and 15 µg of the GFP-transfer vector (Addgene #17451)\textsuperscript{150}. At 24 hours post transfection, 10 mM sodium butyrate was added to the cells. Lentivirus was harvested and concentrated at 48 hours post transfection using Lenti-X Concentrator (Clontech). To transduce sorted RGCs, lentivirus with 8 µg/mL Polybrene (Sigma) was added to the cell culture media overnight at 5-10 multiplicity of infection. The next day, the cells were washed with PBS and cultured with N2B27 media.

2.4H Adeno-associated virus production and transfection of sorted RGCs

AAV2:mitoEGFPmCherry\textsuperscript{151} was produced and purified by triple transfection and heparin column purification\textsuperscript{152}. The AAV cis-plasmid was pENN.AAV.CB7.CI:mito-EGFP-mCherry-SV40pA, the AAV trans-plasmid was p5E18 (pAAV2/2, Penn Vector Core), and pAdDeltaF6 (Penn Vector Core). The protocol from McClure, et al\textsuperscript{152} was followed, except only AAV2 and not AAV1 was used in the transfection. Fluorescence images were taken one week after transfection.
2.4I Nanofiber scaffold preparation and Matrigel droplet culture

Aligned polycaprolactone (PCL) nanofibers were prepared by electrospinning according to a previously reported method. Briefly, PCL (MW 80 KDa, Sigma) was dissolved in hexafluoroisopropanol (HFP, Sigma) to obtain a solution of 17.5 w/v%. This solution was spun at a flow rate of 0.35 mL/hour through a blunt-end 27-gauge needle over a 42-mm separation distance towards a grounded rotating wheel (1,000 rpm), to which 15-mm glass coverslips were attached at a distance of 18 cm from the center or the wheel. A positive charge of 7 kV was applied to the needle tip (Gamma High Voltage Research). The average diameter of the fibers collected under these conditions was 632 nm ± 140 nm, as determined by ImageJ analysis of several scanning electron microscopy (SEM) images. The nanofibers on coverslips were sterilized by soaking in 95% ethanol, and then washed three times with PBS. The nanofiber coverslips were then coated with Matrigel (100 µg/ml) at 37°C overnight before cell seeding.

Differentiated day 40 or older cells were dissociated to clusters by treating with Accumax for 10 minutes combined with gentle trituration. The cell clusters were collected by centrifugation and resuspended in 100 µL of pure ice-cold Matrigel solution. This cell cluster suspension was distributed to dry Matrigel pre-coated plates with nanofiber matrices and incubated at 37°C for 20 minutes to allow the Matrigel droplets to form a hydrogel. The wells were then filled with N2B27 media supplemented with 10 µM Forskolin and 10 ng/mL CNTF and refreshed every other day.

A limited diffusion culture was established to localize growth factor release between two neighboring cell clusters seeded on aligned nanofibers assembled within a
microchannel in order to enhance axon network formation between the two clusters. To prepare the nanofiber coverslips with a limited diffusion channel, nanofibers on the coverslips were trimmed to 10 × 2 mm rectangular sheets with nanofibers aligned along the long axis of the sheet. Both ends of the sheet were immobilized with silicone glue. A custom-made polydimethylsiloxane (PDMS, Sylgard 184) migration chamber consisting of two 3-mm diameter microwells with a connecting channel with dimensions of 10 mm (length) × 2 mm (width) × 100 µm (height) was placed over the nanofiber sheet. 1% Matrigel solution diluted in PBS was pipetted into the microwells and allowed to adsorb for 1 hour, and then washed with PBS before use. Cell clusters were seeded into microwells and then cultured according to the same protocol as described above.

2.4J Calcium imaging

Calcium imaging was performed as described previously\(^{154}\). Briefly, cells were purified by FACS on day 45 and cultured for 6 days before being loaded with 3 µM Fluo-4AM (Life Technologies), in HEPES-buffered physiological salt solution (PSS) containing 0.04% Pluronic F-127 (Life Technologies) for 60 minutes at room temperature\(^{154}\). Cells were washed with PSS 3 – 5 times and then imaged using the EVOS FL Auto Cell Imaging System (Life Technologies). Images were taken every 3 seconds. ImageJ was used to analyze images.
2.4K Quantitative real-time PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) and treated with RNase-free DNase I (Qiagen) to remove genomic DNA contamination. For FACS purified cells, approximately 100,000 sorted cells were used for each RNA extraction sample. Extracted RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qPCR was performed with the CFX384 real-time PCR instrument (Bio-Rad) using the following program: 95°C, 60°C, and 72°C – 40 cycles. All assays included technical and biological triplicates in 10 µL reactions using the Sso Advanced Universal SYBR Green Supermix (Bio-Rad) and 400 nM oligonucleotide primers. Primers used were designed by Geneious R7 (Biomatters) or sequences were obtained from publications or the PrimerBank database (Table 1). Sample gene expression was normalized to the geometric mean of the reference genes GAPDH and CREBBP. Relative normalized expression and the standard error of the mean (SEM) were calculated using Bio-Rad’s integrated CFX manager software.

2.4L Immunocytochemistry

Cells were fixed with 4% paraformaldehyde in 0.2 M Sorenson's phosphate buffer (pH 7.4) for 10 minutes at room temperature, washed three times with PBS, permeabilized with 0.1% Triton X-100 in PBS for 15 minutes, blocked using 2% bovine serum albumin (BSA) or 2% horse/goat serum in PBS for 1 hour at room temperature, and then incubated with primary antibody overnight at 4°C with 0.1% Triton X-100. Primary antibodies used were TUJ1 (mouse, 1:2,000, Covance, MMS-435P), CRX
(mouse, 1:10,000, Novus Biologicals, H00001406-M02), mCherry (rabbit, 1:200, Abcam, ab167453), TAU (chicken, 1:50, Abcam, ab75714), MAP2 (rabbit, 1:200, Santa Cruz, sc-20172), PAX6 (rabbit, 1:500, Covance, PRB-278P), THY1 (mouse, 1:100, Stemcell Technologies, 60045FI), BRN3 (goat, 1:50, Santa Cruz, sc-6026), BRN3A (mouse, 1:50, Santa Cruz, sc-8429), BRN3B (rabbit, 1:2000, Abcam, ab56026), RBPMS (rabbit, 1:50, Santa Cruz, sc-133950), ISL1 (rabbit, 1:100, Abcam, ab20670), NEFH (rabbit, 1:200, Abcam, ab8135), SNCG (mouse, 1:1,000, Abnova, H00006623-M01), and NEUN (mouse, 1:50, Millipore, MAB377). Cells were incubated with species-specific corresponding secondary antibodies for 45 minutes at room temperature. Secondary antibodies used included Alexa Fluor-488 and 647 conjugated antibodies (1:1,000, Life Technologies). Hoechst 33342 (trihydrochloride, trihydrate, Life Technologies) was used to stain nuclei. Fluorescence images were acquired with the Eclipse TE-2000S inverted microscope (Nikon) and the EVOS FL Auto Cell Imaging System. Identical samples, without the primary antibody added, were used as controls for antibody specificity.

2.4M Transmission electron microscopy (TEM)

FACS sorted cells from day 40 of differentiation were seeded on Matrigel coated glass coverslips in 35 mm tissue culture dishes at a density of 50,000-100,000 cells/cm². Cells were fed with N2B27 media containing 2 µg/mL doxycycline, 1 µg/mL mitomycin-C, 10 µM Forskolin, and 10 ng/mL CNTF. After 24-hours, the media was replaced with N2B27 supplemented with Forskolin, CNTF only. Seven days post plating the cells were fixed for 1 hour in 2.5% glutaraldehyde/2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 (all from Electron Microscopy Sciences) at room temperature
and overnight at 4°C. Cells were post-fixed with ferrocyanide (Sigma-Aldrich)-reduced osmium tetroxide (Electron Microscopy Sciences), stained with 1% thiocarbohydrazide (Polysciences, Inc.) followed by 2% osmium tetroxide. Cells were subsequently stained with 1% uranyl acetate (Electron Microscopy Sciences) and 0.66% lead nitrate (Electron Microscopy Sciences) in 0.03 M aspartic acid, pH 5.5 (Sigma-Aldrich) before progressive dehydration into ethanol. Durcupan (Sigma Aldrich) embedded cells were hardened for 36-48 hours at 60-65°C. 60 nm-thick sections were imaged using an H7600 transmission electron microscope (Hitachi).

2.4N Live cell imaging

The EVOS FL Auto Cell Imaging System was used for imaging cells in culture over time and for scanning whole live culture plates. During imaging experiments, cells were maintained in a live cell chamber at 37°C with 5% CO₂ and 85% humidity.
Table 1

qPCR primers used in Chapters 2, 3, and 4

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Mismatch bases are in bold
Chapter 3: Efficient large scale production and affinity purification of human retinal ganglion cells differentiated from pluripotent stem cells.

3.1 Introduction

Retinal ganglion cell (RGC) axons connect the eye to the brain. Death of these cells leads to irreversible vision loss, which can result from trauma or diseases such as glaucoma, the second leading cause of blindness worldwide\textsuperscript{91,92}. Current glaucoma treatment is based on lowering intraocular pressure (IOP) through pharmacological or surgical means, but IOP control is not always achievable and even with significant IOP lowering RGC loss can still progress\textsuperscript{157}. In addition to glaucoma, RGC death occurs in a number of other diseases (reviewed in Levin and Gordon\textsuperscript{158}) such as dominant optic atrophy (DOA) and Leber's hereditary optic neuropathy (LHON), diseases of relatively early onset without an IOP component and no treatment options. In order to develop novel therapies for glaucoma as well as for other RGC diseases, it remains an important goal to identify neuroprotective compounds that promote RGC function and survival\textsuperscript{3}. In addition to the use of model organisms for studies of disease, pluripotent stem cells (PSCs) can also be used to generate cell types of interest in disease modeling efforts\textsuperscript{159-163} and can, at times, reveal insights that are specific to the human system\textsuperscript{163}.

PSCs can be differentiated to retinal cells, including RGCs using a growing number of protocols\textsuperscript{24,25,61,95,99}. Using three dimensional aggregate suspension cultures, it has become possible to form retinal organoids\textsuperscript{24,25} that contain all of the expected retinal layers. When looking for structural developmental phenotypes, these three dimensional organoids provide a useful window into a world of human development that may
otherwise be inaccessible. However, in order to generate cells for large scale experiments such as drug discovery efforts or biochemical analysis, it may be advantageous to differentiate cells using adherent protocols where structural organization may be lost, but RGC yield is maximized per researcher time.

As described in Chapter 2, we have developed an adherent cell protocol for RGC differentiation using a CRISPR-Cas9 generated RGC reporter line where the \textit{BRN3B} (\textit{POU4F2}) open reading frame (ORF) was combined with mCherry via a P2A peptide\textsuperscript{51}. While this cell line has been very useful in establishing a better RGC culture system, it has proven cumbersome as a method for efficiently isolating large numbers of cells by fluorescence activated cells sorting (FACS) due to the extensive time required for this technique. In an effort to develop a simpler and more powerful approach for generating large numbers of highly purified human RGCs, we have worked to improve both the efficiency of differentiation and isolation of RGCs. Here, we report a novel cell isolation scheme based on CRISPR gene editing to generate a BRN3B-P2A-tdTomato-P2A-Thy1.2 reporter line capable of RGC-specific affinity purification. Additionally, using small molecules, we have improved our differentiation culture parameters to generate adherent cultures containing up to 50\% RGCs as a percent of the total population.

3.2 Results

3.2A Generation of a novel BRN3B reporter line

As with our previously generated RGC reporter line, we designed our new reporter using the \textit{BRN3B} gene\textsuperscript{51}. \textit{BRN3B} is a crucial RGC transcription factor and gene marker\textsuperscript{36,37} with desirable reporter qualities of early expression and relative
specificity\textsuperscript{36,37,102}. As before, we chose to combine the reporter gene with the \textit{BRN3B} open reading frame via a P2A self-cleaving peptide\textsuperscript{103} in order to best maintain normal BRN3B expression levels without creating a fusion protein. We substituted mCherry with tdTomato and added a second P2A peptide linking tdTomato to \textit{Thy1.2}, the mouse ortholog of the human \textit{THY1} gene, to create a single ORF of BRN3B-P2A-tdTomato-P2A-Thy1.2. This ORF should produce the BRN3B transcription factor, tdTomato, and Thy1.2 with nuclear, cytoplasmic, and cell surface localization, respectively (Fig. 19a). We exchanged mCherry for tdTomato in order to increase reporter brightness and added Thy1.2 to generate a unique surface antigen that could be used for affinity purification of only BRN3B expressing cells from culture as based on the following observations. We had tested whether human RGCs derived from our BRN3B-P2A-mCherry (A81-H7) line could be isolated by immunopanning\textsuperscript{164} for mouse or human \textit{THY1}\textsuperscript{125}, and we observed that human cells did not bind to the antibody against the mouse version of this protein (Fig. 20a). Additionally, while some fluorescent RGCs did bind to the anti-human \textit{THY1} antibody coated plates, a large number of non-fluorescent cells resembling fibroblasts were also bound and continued to proliferate, leading to a complete loss of RGC enrichment in the cultures (Fig. 20b,c). Therefore, we decided to knock-in the mouse ortholog \textit{Thy1.2} into the \textit{BRN3B} locus to enable only BRN3B+ cells to bind to immunopanning plates coated with anti-Thy1.2 antibodies. As \textit{THY1} is an RGC marker\textsuperscript{125} that we have shown is also expressed in stem cell derived RGCs\textsuperscript{51}, we thought that the addition of the mouse Thy1.2 would be innocuous to these cells.

We used our previously designed gRNA\textsuperscript{51} to target the stop codon of \textit{BRN3B} and added the P2A-tdTomato-P2A-Thy1.2 sequence to the template homology plasmid. The
Figure 19

(a) Schematic illustration depicting reporter design. CRISPR-Cas9 was used to target the stop codon of *BRN3B* in H7 hESCs. A P2A linked tdTomato was added in tandem with a P2A Thy1.2 to the *BRN3B* ORF. Following translation, the BRN3B transcription factor protein is localized to the nucleus, tdTomato to the cytoplasm, and Thy1.2 to the cell surface. (b) PCR test for zygosity. Primers spanning the integration region were used to amplify genomic DNA for comparison between the parental H7 line and the isolated E4-H7 clone. E4-H7 DNA produced one band of expected integration and one wildtype band, indicating heterozygosity of the modified locus. (c) Fluorescence microscopy of day 35 differentiated tdTomato+ cells. Scale bar=100µm.
Figure 20. Immunopanning of differentiated A81-H7 cells.

Phase and fluorescence images of differentiated day 40 A81-H7 cells in immunopanning experiments. (a) Human cells bound to plates coated with anti-human THY1 but not to anti-mouse Thy1.2 (b) Culture of cells bound to anti-human THY1 plates 24 hours after panning. Fluorescent cells are highlighted (white arrows). (c) THY1 bound fraction of cells one week post panning.
Figure 20

(a) Anti-human Thy1

(b) Bound Fraction

(c)
plasmids for the gRNA, Cas9, and the P2A-tdTomato-P2A-Thy1.2 template were electroporated together into the H7 hESC line. Following single cell passaging, seventy-two colonies were screened for reporter integration by PCR. Efficiency of integration appeared lower than with our previous efforts, but one heterozygous clone was isolated as described in the methods and named E4-H7 (Fig. 19b). Reporter gene integrity of the recombined allele was confirmed by sequencing.

3.2B Reporter line differentiation and immunopanning

We used our previously described protocol\textsuperscript{51} to induce differentiation of the E4-H7 cell line (Chapter 2). This protocol relies on extra-cellular cues from Matrigel\textsuperscript{24,25,105} to induce spontaneous retinal differentiation in N2B27 media. As with our BRN3B-P2A-mCherry cultures, we found that tdTomato+ cells first became visible around day 25 of differentiation and more cells continued to emerge over time (Fig. 19c). To test this new reporter line we immunopanned the cells using anti-Thy1.2 panning plates and found that only the fluorescent cells were able to bind to the plates with very few non-fluorescent cells remaining in this purified population (Fig. 21). However, a large percentage of fluorescent cells remained unbound as well (Fig. 21). We assessed the bound population by FACS and determined the purity of the cells to be over 99% (Fig. 22). We calculated the efficiency of purification by determining the percent of tdTomato+ cells that bound to the plates and dividing it by the starting percentage to yield a value of 46.4%. Thus, immunopanning resulted in a very pure population but more than half of the presumptive RGCs were lost during the procedure.
Figure 21. Immunopanning of differentiated E4-H7 cells.

Fluorescent cells bound to anti-Thy1.2 coated plates with very high specificity. The unbound fraction mostly contained non-fluorescent cells and some fluorescent cells of lower intensity. Scale bar=100µm.
Figure 22. Assessment of immunopanning purity by FACS.

Differentiated E4-H7 tdTomato+ RGCs were purified by immunopanning with anti-Thy1.2 coated plates and the unbound and bound fractions were analyzed by FACS. The bound fraction appears very pure and enriched for higher fluorescence intensity cells. Lower fluorescence intensity cells are largely located in the unbound fraction (black arrows).
We then sought to further optimize our differentiation conditions, using either A81-H7 or the new E4-H7 cell line, and were surprised to find that a Matrigel cover layer was not necessary for RGC differentiation as fluorescent cells emerged at similar rates in cultures that were simply plated on Matrigel coated plates (Fig. 16c. Fig. 23a). We also found that Forskolin still improved differentiation in this simplified version of our protocol (Fig. 16c. Fig. 23a, Chapter 2). Additionally, we switched from plating hESC colony clumps in favor of plating single cells as defined cell numbers in the presence of Rho kinase (ROCK) inhibitors which are required for single-cell stem cell survival. This modification allowed us to test and control cell density in future experiments, culminating in the establishment of 52.6K/cm$^2$ as the most potent density for RGC differentiation using the H7 hESC line (Fig. 23b).

3.2C Improved differentiation to RGCs using Dorsomorphin, IDE2, Nicotinamide, Forskolin, and DAPT

We had speculated that implementation of the dual SMAD inhibition protocol described by Chambers et al.\textsuperscript{165} may increase RGC yield as this protocol efficiently generates neural progenitors of an anterior CNS fate\textsuperscript{165}. Thus, we added SB431542 and LDN-193189, a more recent dual SMAD inhibition small molecule combination\textsuperscript{166}, to our differentiation culture. Counterintuitively, we found that SB431542 inhibited retinal differentiation to RGCs, an effect that was more pronounced with addition of LDN-193189 (Fig. 24). Upon further investigation, we noticed that the optic differentiation study of Eiraku et al.\textsuperscript{24} had described one of the dual SMAD inhibitors, SB431542, to
Figure 23. Matrigel cover layer and density effect on RGC differentiation.

FACS analysis for percent of mCherry+ cells (A81-H7 line). Cells were differentiated for 40 days before analysis in the absence of a Matrigel cover layer. (a) Cells were treated with 25 µM Forskolin (FSK) or DMSO from days 1 to 6 of differentiation. P values were 0.0419 and 0.0063, respectively. *p<0.01, **p<0.05. N=3. Unpaired two-tailed t-test was used to compare Forskolin treated samples with DMSO. (b) Cells were plated at an increasing density of 1,000s per well of a standard 24 well plate. (a, b) Error bars represent standard deviation.
Figure 24. Effect of dual SMAD inhibitors SB431542 and LDN-193189 in RGC differentiation.

Fluorescence microscopy scans of differentiated day 37 A81-H7 cultures. SB431542 alone or in combination with LDN-193189 was applied from day 2 to 7 of differentiation.
inhibit Matrigel induced spontaneous retinal differentiation in their protocol. They further
deduced that Matrigel could be replaced by a combination of laminin, entactin, and
Nodal, a member of the TGF-β family that acts through Activin receptors. We
speculated that these principles could be applied to our differentiation protocol and so we
removed SB431542, which inhibits the Lefty/Activin/TGF-β pathway, while retaining
LDN-193189 or using Dorsomorphin (DSM) to inhibit the BMP pathway. Interestingly,
DSM alone also decreased the number of RGCs produced in differentiation (Fig. 25a).
We speculated that this decrease could be due to additional reported activity of DSM
against Activin, a weaker inhibition than the one bestowed by SB431542, but an effect
that could impact Nodal signaling. Furthermore, the endogenous BMP signaling inhibitor
protein, Noggin, failed to increase RGC differentiation as well (Fig. 25c), likely due to its
own reported inhibition of Nodal. To provide supplementary Nodal signaling and
possibly nullify the DSM effect on this pathway, we decided to add IDE2, a small
molecule capable of replacing Nodal in stem cell differentiation. Although IDE2 was
not able to increase RGC differentiation on its own as well (Fig. 25b, Fig. 26a), the
combination of IDE2 and DSM or LDN-193189 increased the amount of fluorescent cells
when added in the first week of culture (Fig. 26a). As the combination of DSM and IDE2
appeared best, subsequent differentiation was done using this combination we termed
DID. Importantly, when we applied the two small molecules together to cultures
differentiated with a Matrigel cover layer, per our prior protocol, DID did not have a
positive effect on differentiation (Fig. 26b), suggesting that Matrigel can unpredictably
interfere with small molecule effects on differentiation.
Figure 25. Effects of DSM, Noggin, and IDE2 on RGC differentiation.

E4-H7 was used for all experiments. (a) DMSO or DSM were applied to differentiation from day 1 to 8. Cells were analyzed on day 40. P value was 0.0258. **p<0.05. N=3. Unpaired two-tailed t-test was used to compare DMSO to DSM. Error bars represent standard deviation. (b) DMSO or IDE2 was applied to differentiation from days 1 to 6. Cells were analyzed on day 43. (c) Whole-well fluorescence microscopy of differentiated day 46 cells treated with recombinant Noggin for days 1 to 6 or a negative control.
Figure 26. Combination of DSM and IDE2 (DID) in differentiation.

(a) Cellomics scan of whole wells of differentiated cells. IDE2, DSM, or LDN-193189 alone or in combinations were applied to cultures from day 1 to 4 or 6. Total fluorescence area was calculated.

(b) Whole-well fluorescence microscopy images of day 30 differentiated cultures treated with DID for days 1 to 6. Cells were differentiated with or without a Matrigel cover layer. A81-H7 was used.
We optimized the timing of addition of DID to the cultures as based on fluorescence, and established that days 1 to 6 worked best for RGC differentiation (Fig. 27). Additionally, we looked for a dose response of DSM, starting with 1 µM as reported previously\textsuperscript{168}, but found any higher doses to be toxic in H7 hESCs. On the other hand, IDE2 was reported to have a rather wide working range\textsuperscript{170}, and we found it to be largely non-toxic without an apparent effect on RGC differentiation with increased concentration (data not shown), perhaps owing to the fact that our starting concentration of 2.5 µM is already saturating as reported\textsuperscript{170}.

Recently, we had identified Forskolin (FSK) as a promoter of RGC differentiation\textsuperscript{51}. Additional experiments revealed that another molecule, Nicotinamide (NIC), implicated in neuronal as well as retinal pigment epithelium (RPE) differentiation\textsuperscript{171-173}, was able to increase RGC differentiation when combined with FSK (Fig. 28). Interestingly, NIC alone did not appear to promote RGC differentiation, needing the presence of FSK for a noticeably beneficial effect. We further tested whether DID would synergize with NIC and FSK together (NF) in a combination we named DIDNF. Indeed, DIDNF generated the largest percentage of reporter positive cells as assessed by FACS (Fig. 29). Encouraged by the growing increases in RGC number we were seeing with this expanding list of small molecules, we decided to test whether the combination of all four molecules (DIDNF) would be able to replace the need for Matrigel in differentiation. Matrigel is an animal product that contains 1,000s of proteins\textsuperscript{174} and exhibits variability\textsuperscript{175}, making it less desirable for standardization of differentiation protocols. We turned to Synthema as a coating replacement as it is an animal-free, synthetic substrate and should not vary\textsuperscript{176-178}. Notably, stem cell
Figure 27. Optimization of DID timing for differentiation.

(a) Whole-well fluorescence microscopy scans of day 37 differentiated E4-H7. (b) FACS analysis of individual wells from (a). DID from day 1 to 6 appears best for RGC differentiation.
Figure 28. Effect of Forskolin in combination with Nicotinamide on differentiation.

Whole-well fluorescence microscopy images of E4-H7 cultures differentiated for 40 days in the presence of DMSO control, FSK, NIC, or NIC with FSK. FSK was added from day 1 to 30 and NIC from day 1 to 10.
Figure 29

FACS analysis of E4-H7 differentiated cultures. DID and IDE2 were added from day 1 to 6, NIC from day 1 to 10, FSK from day 1 to 30. DMSO was used as a control. NF=NIC+FSK. (a) day 41 analysis, (b) day 42 analysis. DIDNF appears to be the most potent combination of factors for RGC differentiation.
Figure 30. Differentiation of reporter lines on Synthemax highlights strong promoting effect of DIDNF for RGC genesis.

Whole-well fluorescence microscopy images of differentiated cells treated with DMSO, DID, IDE2, NF, or DIDNF on Synthemax coated plates. (a) day 41 culture, (b) day 42 culture. (c) FACS analysis of differentiation in (b). P values were 0.0133 and 0.0054, respectively. **p<0.05, *p<0.01. N=3. Unpaired two-tailed t-test was used to compare DMSO to DID and NF to DIDNF. Error bars represent standard deviation. E4-H7 used for all experiments.
differentiation to RGCs was not evident on Synthemax coated plates (Fig. 30), supporting the role of Matrigel in driving retinal differentiation. Remarkably, while DID or NF treatments alone were able to generate a small number of RGCs on Synthemax, the DIDNF combination appeared to fully rescue differentiation (Fig. 30), suggesting that these small molecules do, indeed, synergize together to drive RGC differentiation and can do so in the absence of Matrigel.

As a final small molecule to add to our DIDNF combination, we turned to DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester), a well characterized γ-secretase inhibitor capable of downregulating the Notch signaling pathway to promote neuronal differentiation. Interestingly, while Notch signaling has been implicated in RGC development, and a number of stem cell studies have shown that Notch inhibition by DAPT can increase RGC differentiation, we saw no benefit from DAPT in our initial Matrigel cover layer differentiation protocol cultures (Fig. 31a). A similar observation was made by Nakano et al., who also used Matrigel to induce differentiation, where a photoreceptor effect was evident from DAPT, but RGC number was not increased. However, in our new protocol with only a Matrigel coating without a cover layer, DAPT did have a positive effect on differentiation of RGCs when added from day 18 to 30, a window that we hypothesized would give retinal progenitors maximal time to form and then be biased towards the early established RGC cell fate (Fig. 31b). Moreover, DAPT synergized with DID to further increase RGC yield (Fig. 31b, Fig. 32a). Since our initial DAPT addition timing was based on an estimate, we performed an optimization of DAPT addition from day 12 to 40 with RGC yield assessment on day 40 (Fig. 32b,c, and d). Our initial DAPT addition from day 18 to 30...
**Figure 31. Effect of DAPT on differentiation.**

Whole-well fluorescence microscopy images of differentiated cells treated with DMSO, DID, or a combination with DAPT. DAPT was added from day 18 to 30, DID was added from day 1 to 8. (a) Matrigel cover layer culture, DAPT did not appear to increase differentiation. (b) No Matrigel cover layer culture, DAPT appears to promote RGC genesis with and without DID, day 45 imaging.
Figure 32. Effect of DAPT on differentiation assessed by FACS.

FACS analysis of E4-H7 differentiated cells treated with DMSO, DID, DIDNF, or their combination with DAPT. (a) FACS analysis of cells displayed in Figure 31b. (b) Day 40 differentiated cells treated with standard DIDNF alone and in combination with DAPT for days x to x. (c) and (d) Day 40 differentiated cells treated with DMSO or standard DIDNF in combination with DAPT for days x to x. DAPT treatments for days 18 to 30 appears best. N=3. Error bars represent standard deviation.
Figure 33. Confirmation of DIDNF+D protocol in A81-H7 and E4-H7.

(a) Schematic of the DIDNF+D RGC differentiation protocol. (b) FACS analysis of differentiated day 40 cells from the two different lines, A81 and E4, treated with DMSO or standard DIDNF+D. N=3. Error bars represent standard deviation.
appeared best, however, and thus, our final protocol became DIDNF+D (Fig. 33a). We then validated the full DIDNF+D protocol in the BRN3B-P2A-mCherry stem cell line as well, to confirm that its efficacy was not restricted to E4-H7 (Fig. 33b). While our differentiation percentage varied, the DIDNF+D protocol was able to reliably generate between ~20 to 50% RGCs as compared to the total cell population.

To make sure that our small molecule treatment did not push the cells to differentiate toward another lineage, we tested the tdTomato+ cells for a range of RGC-associated gene markers. Since immunopanning selects only for the highest expressing tdTomato+ cells, we used FACS sorting to isolate more of the entire tdTomato+ population. We then compared tdTomato+/− populations from DIDNF+D and no small molecule control differentiations by qPCR (Fig. 34). We found that DIDNF+D differentiated tdTomato+ cells were very similar to control cells, as both populations expressed the RGC-associated genes of *BRN3A, BRN3B, BRN3C, ISL1, ISL2, NHLH2, OPN4, PAX6, POU6F2*, and *RBPMS*, strongly supporting the RGC cell fate of both populations51. Despite a relatively small subset of genes, differences in expression were evident between the two groups. In the tdTomato+ population, DIDNF+D appeared to decrease *BRN3A* expression, while increasing *POU6F2, PAX6*, and *NHLH2*. These changes in gene expression suggest that preferences for RGC subtype generation may be altered by DIDNF+D to perhaps slightly select for BRN3B/BRN3C+ RGCs over BRN3A. The most striking increase was observed for *NHLH2*, an RGC-associated transcription factor121,180. Unfortunately, RGC subtype distribution or spatial expression of NHLH2 is not known.
Figure 34. qPCR analysis of gene expression of DIDNF+D sorted cells compared to spontaneously differentiated cells.

qPCR analysis of FACS-sorted red and not red cells from standard DIDNF+D treated samples and a control sample differentiated without small molecules (no SMs). Solid red and black bars represent sorted red and not red DIDNF+D treated cells, respectively, clear red and black bars represent cells differentiated without SMs. Cells were sorted on day 40 of differentiation. Gene expression was normalized to GAPDH and CREBBP. Error bars represent SEM. Three biological samples were used for each condition.
3.2D Improved purification of RGCs using Thy1.2 microbeads

After increasing RGC differentiation via our DIDNF+D protocol, we set out to improve RGC purification over immunopanning since its application leads to substantial RGC loss (Fig. 21, Fig. 22). Alteration of immunopanning temperature or anti-Thy1.2 antibody concentration did not increase RGC retention, leading us to explore affinity purification using magnetic activated cell sorting (MACS) MicroBeads technology from Miltenyi Biotec. MACS beads are magnetic and are coupled to antibodies, in our case anti-Thy1.2 (CD90.2). Cells are first incubated with the beads and then run through gravity flow columns in the presence of a magnetic field to retain Thy1.2+ cells while allowing the rest of the cells to flow through. As this technology relies on multiple microbeads binding to target cells, the accessible surface area of a cell for antibody binding should be increased as compared to immunopanning, resulting in higher retention of Thy1.2+ RGCs. Indeed, in stark contrast to immunopanning (Fig. 22), microbead purification resulted in increased RGC retention of ~60-80% while maintaining a similarly high purity (Fig. 35, Table 3). Thus, this technique allowed us to isolate extraordinarily large numbers of pure RGCs in a much shorter amount of time than FACS or immunopanning. Additionally, we observed less cell death after purification with the MACS procedure, over the FACS or immunopanning methods.

3.2E Applications of purified human RGCs

With increased scale and throughput of RGC purification, we first wanted to pursue drug screening as a test of our stem cell-derived human RGC system. However, as we had found previously, stem cell-derived RGCs do not succumb to cell death for
Figure 35

**Figure 35. MACS purification of E4-H7 differentiated RGCs.**

FACS analysis of the MACS procedure for purifying RGCs from culture. The starting population, unbound fraction, and bound cells were analyzed after elution from the MACS column.

- **Before panning:** 10.90% red
- **Unbound fraction:** 2.05% red
- **Bound fraction:** 95.85% red
Figure 36

(a) RGC survival of MACS purified RGCs treated with increasing doses of colchicine.
(b) RGC survival after treatment with 1 µM colchicine in the presence of DLK inhibitors.

This figure was generated by Dr. Derek Welsbie from Johns Hopkins University School of Medicine.

Figure 36. Colchicine induced death of RGCs and their rescue with DLK inhibitors.

(a) RGC survival of MACS purified RGCs treated with increasing doses of colchicine.
(b) RGC survival after treatment with 1 µM colchicine in the presence of DLK inhibitors.

This figure was generated by Dr. Derek Welsbie from Johns Hopkins University School of Medicine.
months without exogenous stimuli\textsuperscript{51}. Therefore, we induced cell death of the purified RGCs using colchicine\textsuperscript{181-183}. We observed a strong decrease in RGC survival with increasing concentration of colchicine within 48 hours (Fig. 36a). Previously, we had established that colchicine induced cell death in primary mouse RGCs can be suppressed through inhibition of dual leucine zipper kinase (DLK) (unpublished data and Welsbie et al.\textsuperscript{3}). Therefore, we tested whether DLK inhibitors could rescue human stem cell-derived RGCs as well. Indeed, administration of known DLK inhibitors along with 1 µM colchicine (a dose capable of inducing near-complete RGC death) resulted in a dose dependent survival increase (Fig. 36b), thus validating the potential use of the human RGC system as a model of RGC death in drug discovery.

We also explored preliminary transplantation experiments of tdTomato\textsuperscript{+} RGCs into the vitreous of mice. We used wildtype mice rather than immune compromised mice for the initial effort, as the eye has been described to be immune privileged\textsuperscript{184}. The mice were sacrificed at one, two, or three weeks post injection. We looked for tdTomato\textsuperscript{+} cells in the retinas of the transplanted mice and were able to find fluorescent cells on the retinal surface at one week post-injection (Fig. 37a). However, none of these cells displayed neurites and the two to three week post-injection retinas did not contain fluorescent cells, suggesting that perhaps the immune system was clearing away the exogenous RGCs over time.

Generally, RGCs have to be utilized in subsequent applications shortly after isolation since they are post-mitotic neurons that can no longer be expanded and multiple cell passaging events often result in neurite damage and cell death. However, we noticed
Figure 37. Transplantation and sphere formation of MACS purified RGCs.

(a) Intravitreal injection of purified human RGCs into mice. Mice were sacrificed 1 week post injection and retinas were imaged using confocal fluorescence microscopy. (b) Plated re-aggregated spheres of RGCs. (c) Frozen RGC spheres, thawed and re-plated for adherent culture. Scale bars=100 µm.
that when RGCs were plated in low binding dishes, they tended to aggregate and form floating spheres. These spheres demonstrated robust neurite outgrowth when plated on regular tissue culture plates which intrigued us as a potential use for studies of axonal outgrowth, myelination, and injury (Fig. 37b). Additionally, we found that these RGC spheres could be frozen and stored for later use or be shipped to other researchers for further study (Fig. 37c).

3.3 Discussion

The use of stem cell-derived cells for research that requires large numbers of cells, such as high throughput drug discovery or biochemical inquiry, is often hindered by a lack of robust differentiation and purification techniques for generating the proper cell types of interest. While differentiated cells have already been utilized in drug discovery platforms, these studies are generally limited to particular cell types such as cardiomyocytes\textsuperscript{185} or mixed neuronal cells\textsuperscript{186}. However, if a rare cell type or a homogeneous culture of a specific subtype was desired, accessibility and availability become difficult issues to solve. For example, RGCs are a medically relevant cell type that presents many opportunities for drug discovery\textsuperscript{187} and stem cell differentiation to RGCs has been well-established\textsuperscript{24,25,51,59,60,95,99}. Yet, RGCs emanate from retinal tissue that forms during forebrain differentiation and their isolation from stem cell culture requires RGC reporter cell lines. Moreover, if FACS sorting is used for RGC isolation, experiments become limited by access to expensive equipment and the considerable time needed to sort high numbers of cells. We have designed a novel RGC reporter stem cell line that allows for large scale affinity purification of differentiated RGCs with very high
purity. Additionally, we have developed a small molecule directed differentiation protocol for efficient production of RGCs in adherent culture that makes possible the use of human stem cell-derived RGCs in many applications, including drug discovery.

We used CRISPR-Cas9 technology to knock in a P2A-tdTomato-P2A-Thy1.2 sequence into the BRN3B ORF, a strategy we utilized previously. A heterozygous cell line generated from H7 hESCs was isolated, named E4-H7, and its differentiation resulted in tdTomato+ fluorescent cells that were capable of selectively binding to immunopanning plates coated with anti-Thy1.2 antibodies. Although immunopanning proved advantageous over FACS, as it generated higher purity cultures in less time, many RGCs were lost during the procedure as they failed to remain bound to the plates. Therefore, we improved upon our affinity purification concept by switching to MACS technology using CD90.2 microbeads. MACS increased our RGC retention rates to ~60-80% without a loss of purity, while providing a way to purify up to one billion RGCs in approximately two hours.

In addition to developing a fast and efficient protocol for stem cell-derived RGC purification, we have also improved differentiation of RGCs using the small molecules Dorsomorphin (DSM), IDE2, Nicotinamide (NIC), Forskolin, and DAPT, a combination we termed DIDNF+D. We added DSM and IDE2 to differentiation based on our hypothesis that their inhibition of BMP signaling along with induction of Nodal signaling, respectively, would lead to increased retinal genesis. We based this hypothesis on the finding that the commonly used neurogenesis inducing dual SMAD inhibition protocol prevented RGC differentiation and that Eiraku et al. had demonstrated that recombinant Nodal could replace Matrigel in their retinal differentiation, a protein that
would be inhibited by the SB431542 molecule used in dual SMAD. Other reports have also shown that inhibition of Nodal/Activin instructs differentiation to proceed toward a caudal identity, i.e. away from the retina\textsuperscript{188}. Moreover, zebrafish and mice with Nodal mutations display defects in eye development such as cyclopia\textsuperscript{189,190} as well as loss of \textit{ath5} (\textit{Atoh7}) expression\textsuperscript{191}, an essential gene for RGC development\textsuperscript{32,34}. Furthermore, recently a role for Nodal/Activin signaling in establishment of the eye field has been described in hESC\textsuperscript{192} and mESC differentiation\textsuperscript{193} and addition of IDE1, another Nodal upregulator molecule like IDE2, has been used to enhance stem cell differentiation to RPE\textsuperscript{194}. Therefore, we thought that removal of SB431542 from the protocol while retaining BMP inhibition by DSM would lead to increased retinal differentiation.

Surprisingly, we noticed that DSM alone decreased RGC differentiation, perhaps due to its weaker activity against Activin/Nodal\textsuperscript{168}. Similarly, IDE2 alone did not increase RGC genesis, possibly due to Matrigel inducing enough Nodal signaling to promote peak differentiation. Strikingly, the combination of DSM with IDE2 (DID) did lead to increased RGC differentiation, suggesting that IDE2-increased Nodal signaling could override the DSM-induced inhibition of this pathway. Although more selective inhibitors of the BMP pathway could potentially improve differentiation over DSM\textsuperscript{195}, DSM performed slightly better than LDN-193189 when combined with IDE2 and recombinant Noggin could not drive differentiation on its own either. Therefore, a combinatorial approach to differentiation we applied here may be the best current approach as it appears to be difficult to exclusively inhibit the BMP pathway.

The timing of addition of DSM and IDE2 also proved crucial for retinal differentiation. During optimization, we found that these molecules impacted RGC
differentiation best when added from day 1 to day 6. Interestingly, it has recently been reported that BMP4 addition to optic cup differentiation cultures increases retinal genesis if added on day 6\(^1\), when further BMP inhibition would likely be detrimental\(^2\), as we also observed. In addition to utilizing the DID small molecule combination to promote differentiation to RGCs, we also observed that these molecules synergized with the previously identified Forskolin\(^5\) and NIC\(^{171-173,194}\) (DIDNF). Notably, together these molecules could induce RGC differentiation in the absence of Matrigel, an outcome that was not possible without them in adherent culture. Moreover, since we used an artificial plate coating, Synthemax, the differentiation protocol became practically xeno-free, i.e. containing no animal products, with only B27 containing bovine serum albumin, that can be replaced with human serum albumin if desired for a truly xeno-free system. Thus, our small molecule directed differentiation protocol allows for the production of RGCs in a completely chemically defined manner that could decrease variability or even be used in clinical applications.

To improve our protocol further, we added the Notch signaling inhibitor DAPT to promote differentiation of retinal progenitors toward RGCs\(^{96,97}\). Importantly, during the initial optimization of our protocol, we removed the Matrigel cover layer that we used for prior differentiation experiments\(^5\), and only retained a Matrigel plate coating. In these conditions, DAPT resulted in an increase in RGC genesis and synergized with DIDNF, thus making the protocol DIDNF+D, and allowing for the production of \(~20\) to \(50\%\) of RGCs as a percent of the total population. Notably, the DAPT-induced RGC increase was nullified by a Matrigel cover layer, an observation that may explain the findings of Nakano et al., where a DAPT effect on RGC differentiation was not detected\(^2\).
Moreover, the DID and NIC small molecules also failed to increase RGC yield when used with a Matrigel cover layer, suggesting that either these small molecules fail to penetrate the Matrigel extra cellular matrix or the proteins found in Matrigel\textsuperscript{174} interfere with the small molecule induced effect on cell signaling.

Although this protocol is based on a hypothesis driven approach and known molecules, its mechanism of action in differentiation remains to be validated and further analyzed. Additionally, we found that compared to differentiation without small molecules, the DIDNF+D protocol induced gene expression changes in the generated RGCs, culminating in increased expression of \textit{POU6F2}, \textit{PAX6}, and \textit{NHLH2}, while decreasing expression of \textit{BRN3A}, perhaps suggesting a change in differentiation preference for RGC subtypes\textsuperscript{198}. Identification and isolation of the different RGC subtypes \textit{in vitro} could become very useful in the context of axonal injury and regeneration modeling, where specific subtypes have been shown to differ in regeneration capacity leading to interesting questions of whether the human system follows these rodent paradigms\textsuperscript{199}.

It has not escaped our attention that similar to \textit{BRN3B}, \textit{BRN3A} and \textit{BRN3C} have Cas9 gRNA target sites around their stop codon with very few predicted off-targets (no less than 3 mismatches), creating an ideal situation for reporter line creation using the method described herein. Using \textit{BRN3A} and \textit{BRN3C} reporters in combination with \textit{BRN3B}, should allow for purification of more of the total RGC population and could be used to test whether human stem cell culture produces BRN3+ RGCs in the same ratios between the three transcription factors as has been reported for mice \textit{in vivo}\textsuperscript{36,102}. Additionally, we have designed an OPN4-P2A-tdTomato-P2A-Thy1.2 reporter for
isolation of intrinsically photosensitive RGCs (ipRGCs)\textsuperscript{130} expressing melanopsin (OPN4), thus allowing for purification of this unique RGC subtype from culture. Recently, we have knocked in the \textit{OPN4} and \textit{BRN3B} reporters into BC1 iPSCs\textsuperscript{200} as we wanted to purify ipRGCs from culture as well as to confirm that our DIDNF+D protocol works in multiple stem cells lines rather than just H7 hESCs, the parental line used to make A81 and E4-H7. Homozygous clones for both reporters were isolated in BC1 cells, which should have stronger affinity for CD90.2 microbeads than the heterozygous E4-H7, resulting in higher RGC purification yield. BC1 reporter line differentiation is still ongoing, but as it may not behave as well for retinal differentiation as H7, the P2A-tdTomato-P2A-Thy1.2 reporters will be integrated into other stem cell lines as well for comparison.

As noted for the colchicine experiments in our cells, it appears that human RGCs die in response to colchicine in a DLK related manner. As such, this stem cell system presents a powerful tool for elucidating the mechanism of DLK signaling in axon outgrowth and RGC death. Colchicine induced death may be combined with drug discovery platforms to find additional players in RGC death as well as the DLK mechanism. Moreover, multiple DLK mutations and reporters may be integrated into stem cells prior to differentiation to further probe the role of this gene in RGC death and axon outgrowth\textsuperscript{201}. This strategy represents a lower hurdle than generating knock-in mice and with improving stem cell CRISPR technology may become much more tangible in the near future. Moreover, with large scale RGC differentiation and purification using our methods, millions of human RGCs can be isolated for biochemical analysis of DLK to find protein interactions and uncover novel drug targets.
We believe that through the methods described in this report, large scale human RGC cultures can be generated routinely for a variety of different applications including: RNA-sequencing, mass spectrometry, transplantation, drug discovery, biochemistry, axon guidance, and studies of development. Additionally, the Thy1 strategy we applied here for purification is not limited to RGCs. As these antibodies appear to be highly species specific, and perform really well with the stem cell differentiation system, theoretically any cell type can be targeted for affinity purification using MACS technology. Moreover, although Thy1 knock-in for reporter purposes appears innocuous, it is worthy of note that Thy1 is expressed throughout the nervous system and the immune system, hence it is endogenously present in a wide variety of cell types already. Other surface protein genes may likely be used as well with this reporter strategy. Lastly, we have shown that pure RGCs can be utilized in cell death drug discovery, transplantation, and can be aggregated to form spheres and frozen for later use, allowing for more collaborations to develop between researchers.

3.4 Methods

3.4A Plasmid construction

The same CRISPR guide RNA (gRNA) plasmid and donor template were used as described in 2.4A with modification of the donor to replace mCherry with tdTomato-P2A-Thy1.2 by Gibson Assembly (NEB) to construct the BRN3B-P2A-tdTomato-P2A-Thy1.2 reporter vector. The Thy1.2 ORF was cloned from C57BL/6 mouse cDNA while tdTomato was cloned from an overexpression plasmid. To construct the \textit{OPN4} targeting vectors, an \textit{OPN4} 3’ end targeting gRNA was cloned into the pSpCas9 (BB)-2A-Puro
(PX459) V2.0 vector (Addgene plasmid # 62988). This gRNA has the following sequence targeting the minus strand of **OPN4**: CAGGATGTAGGACGCCCACT. **OPN4** homology arms of 1,028 and 1,010 base pairs, respectively, were cloned into the previous donor vector by Gibson assembly to replace **BRN3B** homology arms. For both, **OPN4** and **BRN3B**, the gRNA target genomic sequence is destroyed by integration of the reporter into the genome and this sequence is not present in the homology template plasmids.

### 3.4B Reporter line generation

Gene editing of H7 hESCs (WiCell) or BC1 iPSCs was performed as previously described in 2.4B with the following modifications. Electroporation was performed using the Neon Transfection System 10 µL Kit (Invitrogen) according to the manufacturer’s instructions. H7 or BC1 cells were dissociated with TrypLE Express (Life Technologies) treatment for 5 minutes. The resulting cell suspension was diluted 1:10 with DMEM/F12 (Life Technologies) and centrifuged at 150xg for 6 minutes, then following supernatant removal, the cell pellet was resuspended in mTeSR1 (Stemcell Technologies) with 5 µM blebbistatin (Sigma). The cells were counted and 150-250x10³ cells were aliquoted to 1.5mL Eppendorf tubes and centrifuged again at 100xg for 7 minutes. The supernatant was removed and the pellet was resuspended in ice-cold R-buffer containing the plasmids encoding the gRNA, Cas9 (Addgene #41815), and the donor template. This suspension was incubated on ice for 5 minutes before electroporation using the following parameters: voltage 1,100 V; interval 30 ms; 2 pulses. After electroporation, the cell suspension was slowly transferred to mTeSR1 medium containing 5 µM blebbistatin and incubated at room temperature for 20 minutes before transfer onto low growth factor Matrigel (BD
Biosciences) coated dishes. Cells were subsequently cultured until splitting as single cells at a low density of 500 cells per well of a 6-well plate. Seventy-two of the resulting stem cell colonies were individually picked and screened for reporter integration by PCR using the primers described in 2.4B. The genomic region containing the integration site was amplified, therefore showing allele zygosity for the reporter. We found a single reporter integration positive colony, a heterozygous clone we named E4-H7. This clone was further expanded and 20 additional colonies were screened for confirmation of heterozygosity of this cell line. The reporter gene was analyzed by DNA sequencing to confirm its integrity. The reporter negative allele was also sequenced. Cas9 editing resulted in a 20 base pair deletion at the 3’ end of \(BRN3B\) for this allele. As the stop codon was targeted by the gRNA, this deletion resulted in a gain of 49 additional amino acids before the next stop codon for this ORF. This mutation did not seem to affect the stem cells or differentiation, however, as the cells appeared healthy and mimicked A81-H7 differentiation. Off-target and karyotype data is still pending for E4-H7.

3.4C Human ESC maintenance

All stem cells were maintained as described in 2.4C.

3.4D Human ESC differentiation towards RGC lineage

Stem cell differentiation was induced as described in 2.4D with the notable optimizations listed in this manuscript. Briefly, hESCs or hiPSCs were dissociated to single cells using the same TrypLE method as in 3.4B. After cell counting, cells were
plated on Matrigel or Synthemax® II-SC Substrate (Corning®) coated plates at a density of 52.6K/cm² in mTeSR1 with 5 µM blebbistatin. Unless otherwise specified, a Matrigel cover layer was not added to the cultures after plating. The day of plating was designated as day minus 1 (d-1). One day after plating, mTeSR1 was completely exchanged for N2B27 media [1:1 mix of DMEM/F12 and Neurobasal (Life Technologies) with 1× GlutaMAX Supplement (Life Technologies), 1× antibiotic-antimycotic (Invitrogen), 1% N2 Supplement (Life Technologies), and 2% B27 Supplement (Life Technologies)] to start differentiation; this day was designated as day 0 (d0). Small molecules were added to the cells on day 1 (d1), 24 hours after d0. Small molecule addition was done in fresh N2B27 media. Cells were fed with full exchange of N2B27 media every other day unless a small molecule was to be removed or added on that day of differentiation, requiring consecutive daily feeding. The following small molecules were aliquotted as 1000x stocks in DMSO and used at the working concentration in parentheses: Forskolin (25 µM - Cell Signaling Technology), Dorsomorphin (1 µM - R&D Systems), IDE2 (2.5 µM - R&D Systems), DAPT (10 µM - Cell Signaling Technology), LDN-193189 (0.5 µM - Stemgent), SB431542 (10 µM - Sigma). Nicotinamide (Sigma) was resuspended in water at 100x and used at a 10 mM working concentration. Noggin (Life Technologies) was resuspended in 10 mM Acetic Acid with 0.5% BSA for a 1000x stock and used as 100 ng/mL. All small molecules were added as indicated. Specifically, for our DIDNF+D protocol, DID was added from day 1 to 6, NIC from day 1 to 10, FSK from day 1 to 30, and DAPT from day 18 to 30. Differentiation was carried out at 37°C in 5% CO₂/20% O₂.
3.4E Fluorescence microscopy

Fluorescence images were taken using the Eclipse TE-2000S inverted microscope (Nikon) or the EVOS FL Auto Cell Imaging System. The EVOS FL Auto Cell Imaging System was used for scanning whole well live culture plates. During imaging experiments, cells were maintained in a live cell chamber at 37°C with 5% CO₂ and 85% humidity.

3.4F Cellomics analysis

To quantify the total fluorescence area induced by small molecule treatment during differentiation, whole well images were taken with a Cellomics ArrayScan VTI HCS Reader (Thermo Fisher), and quantified using the Cellomics Profiling bioapplication.

3.4G Fluorescence-activated cell sorting (FACS)

FACS was performed as described in 2.4E. Threshold of tdTomato fluorescence was the same as mCherry.

3.4H Immunopanning of human RGCs

Immunopanning was performed as described in Welsbie et al³. Briefly, differentiated cells of day 35 or older were dissociated in the same manner as for FACS analysis and then immunopanned on plates preconjugated with anti-Thy1.2 antibody (Serotec, MCA02R) or anti-human Thy1 antibody (Millipore, F15-42-1) and goat anti-
mouse IgM (Jackson Immunoresearch) at room temperature. After washing, bound cells were removed from the plate by a cell lifter and cultured on Matrigel coated dishes in N2B27 media. FACS analysis was used to analyze RGC number before and after panning as based on previously determined fluorescence threshold to assess binding efficiency and purity of the resulting population.

3.4.1 MACS purification of human RGCs

To prepare cells for MACS purification, differentiated cultures of day 35 or later were dissociated to single cells as described for FACS analysis in 2.4E. All reagents were purchased from Miltenyi Biotec. Cells were counted and centrifuged again at 150xg for 8 minutes. For cell numbers less than 50 million, MS columns were used, while LS columns were used to purify larger numbers of cells. The resulting pellet was resuspended according to Miltenyi Biotec instructions in MACS buffer composed of phosphate-buffered saline (PBS), pH 7.2, 0.5% BSA, and 2 mM EDTA made by diluting MACS BSA Stock Solution 1:20 with autoMACSTM Rinsing Solution. The instructed amount of CD90.2 (Thy 1.2) MicroBeads were added to the cell suspension and incubated at room temperature for 15 minutes. This suspension was diluted 1:10 with MACS buffer and centrifuged as before. The resulting pellet was resuspended and added to the prepared MS or LS columns with the attached 30 µm preseparation filters. Mini or Midi MACS separators were used depending on column size. To increase RGC purity to values approaching 99%, cells were generally run through one LS column followed by an MS column without additional supplementation of MicroBeads. Purity was assessed by FACS using the previously determined fluorescence threshold.
3.4J Quantitative real-time PCR analysis

qPCR analysis of gene expression in FACS-sorted RGCs was performed as described in 2.4K. To generate RNA from sorted cells, 100-300x10³ sorted cells were used for each extraction. Primers from Table 1 were used.

3.4K Colchicine induced death

MACS purified RGCs of day 40-50 of differentiation were plated in Matrigel or laminin (Sigma) coated 384 well plates in N2B27 media. To induce death, 1 µM colchicine (Sigma) in PBS was added to the media. Forty-eight hours after addition of colchicine, cell survival was assessed by CellTiter-Glo (Promega) luminescence according to the manufacturer’s instructions. Sunitinib (LC Lab) or Tozasertib (Biovision life science) was added along with colchicine to promote survival.

3.4L RGC transplantation

MACS purified RGCs from day 40-50 were intravitreally injected into adult C57BL/6 eyes using a Hamilton syringe. One to three weeks post injection, mice were sacrificed, retinas were dissociated and fixed in 4% PFA. Confocal microscopy (Zeiss LSM 510) was used to assess cell integration into the retina.

3.4M RGC sphere formation and freezing

MACS purified RGCs were transferred to Ultra-Low Attachment 6 well plates (Corning) at a density of 300-500x10³ cells per well in N2B27 media. RGCs
spontaneously aggregated in 24 hours. Cells were grown in suspension for 48 hours before plating on Matrigel coated dishes or freezing. To freeze RGC spheres, we used Cryostore 10 (Stemcell Technologies) according to the manufacture’s instructions, taking care not to disturb the RGC aggregates as much as possible. Following freezing, RGC spheres could be thawed and plated on Matrigel coated dishes as before.
Table 3

FACS analysis of MACS purifications

<table>
<thead>
<tr>
<th>Pre-binding percent red</th>
<th>Unbound percent red</th>
<th>Bound</th>
<th>Percent retained from first column</th>
<th>Percent red after purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.37</td>
<td>3.92</td>
<td>8.45</td>
<td>68.31</td>
<td>97.02</td>
</tr>
<tr>
<td>6.08</td>
<td>2.35</td>
<td>3.73</td>
<td>61.35</td>
<td>97.94</td>
</tr>
<tr>
<td>10.9</td>
<td>2.05</td>
<td>8.85</td>
<td>81.19</td>
<td>95.85</td>
</tr>
<tr>
<td>22.1</td>
<td>8.73</td>
<td>13.37</td>
<td>60.5</td>
<td>95.56</td>
</tr>
</tbody>
</table>

Bound = pre-bind – unbound

Percent retained = (bound/pre-bind)x100
Chapter 4: Direct reprogramming approaches to RGC generation

4.1 Introduction

Generation of human RGCs in vitro may be accomplished via multiple methods. In addition to stem cell differentiation, as described in Chapters 1-3, RGC-like cells can also be generated via direct reprogramming or conversion of somatic cells to the RGC fate. This direct reprogramming technique is based on transgenic overexpression of genes from cell type “A” into another cell type “B” in order to reprogram or convert cell B to A. Generally, cell lineage specific transcription factors are used in direct reprogramming. For example, exogenous expression of MyoD, a basic helix-loop-helix (bHLH) transcription factor, can reprogram fibroblasts to myoblasts. Similarly, reprogramming to the macrophage cell fate can be induced by C/EBPα/β transcription factors in B cells and by PU.1 and C/EBPα/β in fibroblasts. Likely the most noteworthy direct reprogramming event is the conversion of fibroblasts to induced pluripotent stem cells (iPSCs) using the Yamanaka factors Oct3/4, Sox2, c-Myc, and Klf4. Building on these earlier reports, the reprogramming field accelerated immensely after the demonstration that fibroblasts could be converted to functional neurons with expression of just three factors: Ascl1, Brn2, and Myt1. Subsequent studies were able to show that direct reprogramming could generate a wide variety of cell types including neurons, astrocytes, oligodendrocytes, neural stem cells, cardiomyocytes, hepatocytes, chondrocytes, pancreatic cells, hematopoietic cells and others using an emerging number of methodologies involving transcription factors, miRNAs, and small molecules (reviewed in Xu et al.). Encouraged by these reports, we became interested in whether somatic cells could be directly reprogrammed to RGCs. Using the human retinal pigment...
epithelium (RPE) cell line ARPE19 as our starting cell population to test for genes that could bring about an RGC-like phenotype, we identified a set of four genes ATOH7, BRN3B, MYT1, and BRN2 as necessary and sufficient to reprogram ARPE19 to an RGC-like state. Additionally, expression of these genes in stem cell differentiation increased RGC yield, an effect that was further enhanced with combined Forskolin treatment.

4.2 Results

4.2A Identification of factors capable of ARPE19 to RGC reprogramming

We chose to use ARPE19 as the starting cell population for RGC reprogramming because RPE cells and RGCs both develop from the optic vesicle and would likely be more similar epigenetically than RGCs and fibroblasts, leading to more efficient reprogramming. Additionally, previous reports have shown that RPE cells derived from chick embryos could be reprogrammed to an RGC-like phenotype via overexpression of ATOH7 and NHLH1. We first tested whether ARPE19 were amenable to neuronal reprogramming using the four factors capable of directly converting human fibroblasts to neurons: NEUROD1, BRN2, ASCL1, and MYT1L (NBAM). We were encouraged to find that ARPE19 cells transduced with these factors robustly converted to neuronal cells (Fig. 38). Then, we compiled a list of twelve candidate factors for inducing reprogramming to RGCs: ATOH7, ISL1, BRN3B, BRN3C, TLE1, MYT1, MYT1L, BRN2, SOX4, SOX11, GAP43, YWHAH. These genes were chosen based on their reported roles in RGC development or enriched expression in RGCs. We transduced ARPE19 with a pool of inducible tet-ON lentiviruses carrying these individual genes as
Figure 38. ARPE19 convert to neuronal-like cells in the presence of doxycycline.

(a) Phase microscopy of untransduced doxycycline treated cells. (b) Immunofluorescence microscopy of cells transduced with NBAM factors and stained for neuronal markers TuJ1 and MAP2 after 19 days of doxycycline treatment. Scale bar = 100 µm.
well as the rTATTA. After one week of doxycycline treatment, cells with neurite-like processes became visible (Fig. 39a). We observed considerable cell death in the cultures, but a portion of the surviving cells continued to grow processes over time and transform to a more neuronal-like phenotype (Fig. 39b). We stained the cultures for MAP2, a differentiated neuronal marker\textsuperscript{214}, and observed its expression in the neuronal-like cells (Fig. 39c). We also stained these cells for BRN3A, and observed wide expression of this RGC marker, but we later realized that this antibody cross-reacts with the exogenously added BRN3C. Additionally, the cells appeared to have become post mitotic, as the cultures did not noticeably proliferate further.

4.2B Narrowing down the set of twelve factors to four

We wanted to test whether all twelve of the RGC-associated genes were necessary for the reprogramming of ARPE19 to neuronal cells. We first used the “twelve minus one” approach of leaving one gene out from the set to be used in the conversion and assayed the resulting population by qPCR. Despite the population being heavily skewed toward non-neuronal cells, we did detect a striking decrease in gene expression of THY1, a known RGC marker\textsuperscript{125}, when the transcription factor MYT1 was left out from the set (Fig. 40a). Additionally, when ATOH7 and BRN3B were removed together, there was a decrease in SNCG, NEFH, BRN3A, and endogenous (non-lentivirus mediated) BRN3B expression (Fig. 40b). However, removal of other markers one at a time yielded very little difference in gene expression. Therefore, we began to remove groups of genes together and profiled the transduced cells for continued presence of morphologically
Figure 39. ARPE19 transduced with the set of twelve factors.

Phase and immunofluorescence microscopy of ARPE19 cells undergoing direct reprogramming. (a) No virus control and transduced cells treated with doxycycline for 8 and 16 days, respectively. (b) Higher magnification images of transduced cells after 21 days of doxycycline treatment. (c) Immunostaining for MAP2 and BRN3A in transduced cells treated with doxycycline for 16 days. Scale bars = 100 µm.
Figure 40. qPCR analysis of gene expression in ARPE19 cells undergoing reprogramming with one of the twelve genes taken out.

(a) Analysis of gene expression in ARPE19 cells transduced with the set of twelve genes compared to sets of 11 genes with one gene taken out of the set. From left to right the following genes were taken out: -BRN2, -BRN3B, -BRN3C, -GAP43, -ISL1, -ATOH7, -MYT1, -MYT1L, -SOX11, -SOX4, -TLE1, -YWHAH. Gene expression was normalized to CREBBP and then normalized to ARPE19 cells without virus. Removal of MYT1 from the set of twelve resulted a strong decrease in THY1 expression.

(b) Analysis of gene expression in ARPE19 cells transduced with the set of twelve genes compared to cells without ATOH7 and BRN3B viruses. Gene expression was normalized to CREBBP and GAPDH and then normalized to ARPE19 cells without virus. BRN3B-UTR recognizes endogenous BRN3B but not lentivirus-mediated BRN3B.

Error bars represent SEM. Three biological samples were used for each condition.
neuronal-like cells. These efforts narrowed down the set of needed factors from the initial twelve down to four: *ATOH7, MYT1, BRN3B, BRN2*. We profiled gene expression of cells transduced with four or twelve genes and saw no loss in RGC-associated markers (Fig. 41a). We tried to reduce this set of factors further, and noticed that with combinations of only three genes neuronal-like cells did appear, but there was a decrease in the number of such cells that we could find on the plate. Similarly, qPCR analysis of these three set combinations as compared to four revealed that expression of some RGC-associated markers did not require all four members, but all four factors were necessary for enrichment of the entire panel of markers overall (Fig. 41b). For example, exclusion of *MYT1* or *ATOH7* resulted in *BRN3A* enrichment, but a loss of *THY1*, while exclusion of *BRN2* increased *BRN3A* and *SNCG* expression and decreased endogenous *BRN3B*, *ISL1*, and *NEFH*. Therefore, we concluded that all four of these factors were necessary and sufficient for the conversion of ARPE19 cells to an RGC-like phenotype, as demonstrated by a neuronal morphology and increased RGC-associated gene expression.

4.2C Creation of transgenic ARPE19 lines capable of doxycycline induced reprogramming

As our direct reprogramming system utilizes inducible tet-ON lentiviruses for gene delivery, we decided to select for ARPE19 cells carrying all four identified factors that would convert to RGC-like cells only in the presence of doxycycline. Toward this goal, we combined *MYT1* and *ATOH7* cDNAs into one open reading frame (ORF) and combined *BRN2* with *BRN3B* into another ORF using self-cleaving P2A peptide
Figure 41. Reduction of set of twelve genes to four.

(a) qPCR analysis of ARPE19 cells transduced with the set of twelve or four genes (ATOH7, BRN3B, BRN2, MYT1). Note, ISL1 is present exogenously in the set of twelve genes. (b) Gene expression analysis with one gene taken out of the set of four. From left to right the following genes were taken out: -BRN2, -BRN3B, -ATOH7, -MYT1.

Gene expression was normalized to CREBBP and GAPDH and then normalized to ARPE19 cells without virus. Error bars represent SEM. Three biological samples were used for each condition.
Figure 42. Inducible direct conversion ARPE19 line.

Phase microscopy images of ARPE19 line carrying tet-ON ATOH7, MYT1, BRN2, and BRN3B. Doxycycline treatment for 10 days followed by 5 days of no treatment resulted in ARPE19 cells converted to a neuronal-like phenotype. Scale bars = 100µm.
sequences\textsuperscript{103}, i.e. MYT1-P2A-ATOH7 and BRN2-P2A-BRN3B. Then we cloned these genes into two different lentivirus constructs carrying either puromycin or neomycin resistance genes. Since our rtTA gene-carrying lentivirus uses blasticidin resistance, we could now select for cells definitively transduced with all three viruses, and therefore, select for cells capable of producing separate ATOH7, MYT1, BRN2, and BRN3B proteins after doxycycline treatment. Following transduction and selection with the three antibiotics, a normally dividing transgenic population was isolated; upon addition of doxycycline, a large number of the cells died while many of the remaining cells appeared to be reprogrammed to the RGC-like phenotype (Fig. 42). However, when we profiled these cells for electrophysiological responses typical of neurons, we detected no calcium transients and current injection was not able to elicit action potentials in these cells. Thus, despite the four factors conferring RGC-like gene expression to ARPE19, the cells were not functional neurons.

4.2D Stem cell differentiation using transgenic lines carrying the four factors

Since the four factors we identified were able to induce RGC-like gene expression in ARPE19, we were curious as to whether these factors would also be able to direct stem cell differentiation to RGCs more efficiently as has been described for neuronal reprogramming factors\textsuperscript{213,215}. We transduced the four P2A linked factors and the rtTA into our previously described BRN3B-P2A-mCherry RGC reporter hESC line (A81-H7)\textsuperscript{51} and selected for positively transduced cells. Much like ARPE19, in the absence of doxycycline these cells proliferated normally. Unlike ARPE19 cells, however, treatment of these cells with doxycycline did not result in growth arrest and accelerated
differentiation. The majority of the cells kept dividing, and no fluorescent cells appeared in the first three weeks, suggesting that these factors could not directly induce BRN3B+ RGCs to form. However, if the cells were differentiated per our standard protocol, we saw an increase in fluorescent cells produced when doxycycline was supplemented to the media (Fig. 43a,b). Additionally, when Forskolin was added to differentiation along with transgene overexpression, we saw a more robust increase in generated BRN3B+ cells as compared to doxycycline alone (Fig. 43a,b). This effect was further enhanced when cells were differentiated starting from a single-cell plated culture, rather than clump differentiation as we had used originally (Fig. 43c).

4.2E Attempted conversion to RGCs using Ascl1, Ngn2, and Brn3b

Recently, direct conversion from mouse fibroblasts to RGCs using Ascl1, Ngn2, and Brn3b was reported. We attempted to replicate this conversion event in ARPE19 and the ASCL1, NGN2, BRN3B combination performed worse than ATOH7, BRN3B, BRN2, MYT1 as assessed by qPCR for RGC-associated genes (Fig. 44), an observation that could perhaps be explained by differences in the species and starting cell types used for conversion.

4.3 Discussion

The advent of direct reprogramming of fibroblasts to neurons ushered in renewed interest in this concept that has advanced rapidly in the past few years, as more types of cells have been created with less factors and small molecule supplementation
Figure 43. Direct conversion identified genes drive stem cell RGC differentiation.

(a) Whole-well fluorescence microscopy images of stem cells transduced with the four direct conversion genes and differentiated to RGCs using clump differentiation as described in Chapter 2. Cells were imaged on day 34 of differentiation. FSK was used for days 1 to 6. Dox was added from day 0 to 31. (b) FACS analysis from cultures in (a) performed on day 40. Error bars represent standard deviation. (c) Whole-well fluorescence microscopy images of this transgenic stem cell line differentiated using a single-cell method rather than clump differentiation. Imaged on day 42. FSK was added from day 1-36 and Dox from day 0 to 22.
Figure 44

qPCR analysis of ARPE19 cells transduced with the set of four genes (ATOH7, BRN3B, BRN2, MYT1) or ASCL1, NGN2, and BRN3B. Cells were treated with doxycycline for 15 days before analysis. Gene expression was normalized to GAPDH and then normalized to ARPE19 cells without virus. Error bars represent SEM. Three biological samples were used for each condition.
has replaced all factors entirely in some cases. Here, we report that an established RPE cell line, ARPE19, can be reprogrammed to an RGC-like phenotype using four transcription factors: *ATOH7, MYT1, BRN2, BRN3B*. These reprogrammed cells stain for neuronal markers and express RGC-associated genes by qPCR. Following successful conversion to this RGC-like state, we generated ARPE19 cell lines carrying the four factors in a tet-ON inducible state along with a constitutive rtTA. These cells continued to proliferate, but could be induced to convert to the RGC-like state with doxycycline. Nevertheless, despite their RGC-like gene expression, the cells were not true functional neurons, as evidenced by their inability to fire action potentials spontaneously and in response to current, arguing against their usefulness in RGC modeling as they do not represent a functional phenotype copy. However, the only immortalized RGC line currently available is RGC-5, a discredited cell line of mouse origin that also does not fire action potentials. Moreover, ARPE19 cells converted to RGC-like cells appear to express more of RGC associated genes than RGC-5, suggesting that they may be a better model substitute for real RGCs in certain applications, such as studies of the RGC transcription network.

Despite the advancements in direct conversion methods, it is also worthwhile to consider notable limitations. For example, directly converted cells appear to model their *in vivo* counterparts less closely than stem cell derived cells. Therefore, while ESC or iPSC derived cells may represent embryonic cell types that have trouble modeling late onset diseases, directly converted cells generally retain a large percentage of their epigenetic memory leading to incomplete phenotype mimicry. Moreover, direct conversion tends to produce desired cells at a low efficiency, an issue that can
become problematic when the targeted cell type is a post-mitotic neuron. Although direct conversion efficiency can be increased with small molecules\textsuperscript{227}, these cocktails generally have to be empirically elucidated for each target cell type. Thus, while the direct reprogramming/conversion field continues to innovate, issues remain that must be taken into account before pursuing this experimental direction.

Preeminently, the four factors described in this report were able to increase the number of RGCs formed during normal differentiation. We transduced our previously generated BRN3B-P2A-mCherry RGC reporter hESC line\textsuperscript{51} with the four factors along with the rtTA and applied our standard differentiation protocol\textsuperscript{51}. In the presence of doxycycline, more RGCs were generated, an effect that was further enhanced by Forskolin treatment. It would interesting to see whether the other small molecules that we described in Chapter 3 further synergize with transgene driven differentiation. Although the introduction of transgenic material may be undesirable, if the number of RGCs in differentiation increased dramatically or the culture time needed for differentiation was decreased, it could become a practically useful and scientifically interesting addition to the RGC generation system.

Besides the potential use of transgene factors to drive stem cell differentiation, the reporter technology we described in Chapters 2 and 3 offers yet another application. One of the problems we faced with developing the direct conversion method for making RGCs was the absence of a reporter read-out. Our scoring of reprogramming was largely based on morphology that was later confirmed by qPCR of the entire population. However, if CRISPR tools\textsuperscript{228} could be used to make a \textit{BRN3B} reporter in ARPE19, then we could readily screen for additional factors that could improve this reprogramming
event. Moreover, the stem cell RGC reporter lines we described can also be differentiated to fibroblasts or RPE cells before transduction with lentiviruses for assessment of direct conversion to the RGC fate while using an already available reporter system\textsuperscript{229}, perhaps yielding a different combination of factors then the one we described here.

Lastly, while we were pursuing this project, it was reported that direct conversion from mouse fibroblasts to RGCs could be induced by Ascl1, Ngn2, and Brn3b\textsuperscript{216}. The RGC phenotype in this study was supported by positive immunostaining for Pou6f2, Ath5 (ATOH7), Brn3a, and Thy1.2 in a very small percentage of cells (<0.5%). In our opinion, the data presented in this report is not convincing for RGCs as the immunostaining data does rule out other cell types since Thy1.2 is expressed in the starting fibroblast population\textsuperscript{129} and Ath5 is expressed in many retinal progenitors\textsuperscript{112}. Furthermore, the presence of Ascl1 in reprogramming should technically steer the cells toward a non-RGC lineage according to the literature\textsuperscript{230}. Notably, Ascl1 was essential to reprogramming in this report, and as this factor can induce fibroblast conversion to neurons on its own\textsuperscript{231}, it remains important to validate that these induced RGCs are produced only in the presence of the Brn3b and Ngn2 as well, which could be done by staining cells transfected only with Ascl1 for RGC-markers as a control. Ultimately, the presence of the RGC cell fate would be better supported by qPCR gene expression analysis of a larger cohort of RGC-associated genes and perhaps the presence of long axons expected of RGCs, as we have reported. Nevertheless, we attempted to replicate this conversion event in ARPE19 and the ASCL1, BRN3B, NGN2 factors failed to upregulate RGC associated gene expression to the same level as the ATOH7, BRN3B, BRN2, MYT1 combination.
4.4 Methods

4.4A Plasmid construction

Genes used in reprogramming were cloned from full-length human cDNA clones from Invitrogen's Ultimate ORF collection accessed via the Johns Hopkins HIT Core. Gateway cloning (Thermo Fisher Scientific) was used to insert cDNAs into lentivus destination transfer vectors for the tet-ON 3G system (Addgene #27565, #27566). The reverse tetracycline transactivator 3G (rtTA3G) lentivirus was used for doxycycline inducible expression (Addgene #26429). BRN3B (POU4F2) was cloned from human retina cDNA using Directional TOPO cloning (Thermo Fisher Scientific). SOX4 cDNA was purchased from Open Biosystems (Clone Id: 6584346). To generate MYT1-P2A-ATOH7 and BRN2-P2A-BRN3B, MYT1 with ATOH7 and BRN2 with BRN3B were joined by P2As using Gibson assembly (NEB) and Gateway cloned into neomycin or puromycin resistant lentivirus plasmids.

4.4B Lentivirus production and transduction of cells

Third generation lentiviruses were produced as described in 2.4G with tet-ON transfer vectors. To transduce ARPE19 or human ESC cultures (A81-H7), lentivirus with 8 µg/mL Polybrene (Sigma) was added to the cell culture media overnight at a 5-10 multiplicity of infection for each separate lentivirus. For ARPE19, a single transduction was used while for stem cells, three daily transductions were applied. For inducible cell line creation, ARPE19 or A81-H7 were cultured in the presence of 2 µg/mL puromycin (Sigma), 3 µg/mL blasticidin (Corning Cellgro), and 600 µg/mL G418 (Agilent Technologies) for 4-7 days.
4.4C Cell culture

ARPE19 (ATCC) were cultured in DMEM/F12 (Life Technologies) with 10% Fetal Bovine Serum (FBS), 1× antibiotic-antimycotic (Invitrogen), 1 mM Sodium Pyruvate (Life Technologies), and 1× MEM Non-Essential Amino Acids (Life Technologies). When undergoing direct reprogramming, ARPE19 were cultured in N2B27 media (described in 3.4D) with 2 µg/mL doxycycline (Sigma). For human ESC maintenance, the cells were cultured in mTeSR1 (Stemcell Technologies) as described in 2.4C. The cells were differentiated as clumps as described in 2.4D and as single cells as described in 3.4D. To induce transgene driven differentiation, 2 µg/mL doxycycline was supplemented to the media.

4.4D Immunocytochemistry

Immunocytochemistry was performed as described in 2.4L.

4.4E Quantitative real-time PCR

qPCR was performed as described in 2.4K. To discern endogenous and lentivirus mediated BRN3B expression, primers were designed to target the 3’ BRN3B untranslated region (UTR). The UTR is not present in the lentivirus introduced ORF. Primer sequences for the 3’ BRN3B UTR:

Forward: 5-CCCTTTTCTCGTCCGCTCTTTTC-3
Reverse: 5-GGACTGAAGAGGGAGCGAACGA-3
Amplicon size: 119

CFX manager software was used to graph qPCR data.

4.4F Calcium imaging and electrophysiology

Calcium imaging and electrophysiology were performed as described in 2.4J and 2.4F, respectively.

4.4G Fluorescence microscopy scans

Fluorescence microscopy scans were performed as described in 3.4E.

4.4H Fluorescence-activated cell sorting

FACS was performed as described in 2.4E.
Chapter 5: General discussion and future possibilities

My thesis work has focused on designing technologies and developing methods to generate human RGCs to be used for various applications. This project steered me first, toward the initially exciting and developing field of direct reprogramming by virtues of being brand new technology of unknown potential and the past difficulty of differentiating stem cells to RGCs. However, as we found through a long process of trial and error, direct reprogramming is a difficult technique to implement and requires a well-developed research plan that we were lacking. Nevertheless, I do feel that these efforts yielded more than simply a learning experience, as the four factors that I identified do promote RGC differentiation in ARPE19 and stem cells, and although other factor combinations for this process are likely waiting to be discovered, now we have a robust reporter system that could make this project far more feasible if pursued further. Below I provide more parting thoughts and insights for discussion about this conversion project as well as exciting uses for the stem cell and genome editing technology I have developed.

For direct conversion, it should be noted that we attempted many different gene combinations in addition to the set of twelve described in Chapter 4. While the twelve factors were our first successful combination that resulted in ARPE19 transforming to a neuronal like morphology, we also tested \textit{KLF4, KLF7, NELL2, NRG1, SOX2, FBXO2, BARHL1, IRX2, IRX3, IRX6, NHLH1, PAX6, NGN2, IKAROS}, and other genes in pools of various combinations. Additionally, we attempted to combine miRNA-124 and shRNAs against \textit{PTBP} to further improve reprogramming as has been reported\textsuperscript{232-234}, but
observed no improvement in RGC fate induction as based on morphology or gene expression analysis. Moreover, we supplemented a number of small molecules to the media in attempts to improve reprogramming, including various permutations of: Forskolin, LDN-193189, Dorsomorphin, IDE2, Nicotinamide, SB431542, CHIR 99021, Fenretinide, 5-Azacytidine, Sodium butyrate, Valproic acid, RG108, and Tranyleypromine hydrochloride. However, only Forskolin appeared to promote consistent changes in morphology, which is why it was constitutively added to the direct conversion media and why it was later tried in stem cell differentiation. Lastly, we also tested reprogramming of IMR90 embryonic fibroblasts as well as adult fibroblasts, and while we were able to generate TUJ1+ neurons using published combinations of factors and our set of four as based on immunostaining, we were not successful in inducing the RGC fate from fibroblasts as assayed by BRN3A and/or ISL1 expression. The issue with screening small molecules or transcription factors for a direct conversion event in a system without a reporter is that the number of successful conversion events is usually small, and the time required for conversion is undefined, i.e. how many days of culture must pass before analysis. Therefore, although high-throughput qPCR or immunostaining could be applied here as a screening platform, the signal to noise value may be too low and this approach would likely be a large empirical endeavor. However, with BRN3B reporter lines, regardless of ARPE19 or stem cell origin, the readout becomes far simpler and can be implemented in high throughput FACS analysis or automated imaging systems.

One of the most revolutionary advancements for our stem cell differentiation work has been the serendipitous development of genome editing technologies. These
technologies allowed us to make a number of RGC reporters including the BRN3B-P2A-mCherry and BRN3B-P2A-tdTomato-P2A-Thy1.2 lines described in previous chapters. The use of RGC reporter lines made optimization and development of differentiation protocols possible, such as the DIDNF+D small molecule driven differentiation (Chapter 3). Additionally, with the Thy1.2 affinity purification technique, we can now purify RGCs from culture in a large scale, efficient, and accelerated manner that is not currently available in other stem cell neuronal systems. The advantage of this purification paradigm is highly significant. While FACS purification can be used to generate cells for very small drug screens, the yield and the purity is far inferior to the MACS technique, not to mention the much longer time required to generate an equal number of pure RGCs by FACS. Drug discovery can also be combined with specific RGC mutations. For example, the E50K mutation of optineurin (OPTN) could be knocked-into the reporter line and those RGCs could then be used for drug screening in the E50K background, perhaps leading to medically or biologically relevant insights for this gene and the E50K-associated normal tension glaucoma. Moreover, we have recently performed the MACS purification technique in the absence of bovine serum albumin (BSA) while retaining very high RGC purity. The absence of BSA is noteworthy because it can interfere and contaminate mass spectrometry analysis. Now we can realistically determine the entire proteome of human RGCs in normal as well as stressed states. For example, the proteome of RGCs could be compared with and without colchicine to establish the protein response to this DLK-mediated death, and most interestingly, perhaps we can analyze DLK for post-translational modifications in response to stress to look for a form of DLK that instructs death and another that instructs axonal outgrowth.
Genome editing technologies are novel and still very actively growing, and with expanding knowledge we are able to refine our methods and understanding. As such, it is important to keep some concepts in mind regarding the creation of a knock-in line using these tools. CRISPR-Cas9 as well as Transcription Activator-Like Effector Nucleases (TALENs) create double strand breaks in DNA that can be repaired by homology directed repair (HDR), non-homologous end joining (NHEJ), or microhomology-mediated end joining (MMEJ). A successful knock-in line can be heterozygous or homozygous, but a heterozygous line may have inherent problems. In past techniques that relied on spontaneous HDR to create knock-in mice, it was entirely possible to generate an mESC line carrying only one integrated allele, while retaining a wild type allele as well. However, with double strand break induction, this is not the case. Consider, that if one allele is cut by Cas9/TALEN, chances are the other allele will be, too. Then, if a line is homozygous, we can interpret the event as both alleles were repaired by HDR, but if the line is heterozygous, then one allele was repaired by HDR while the other was repaired by NHEJ or MMEJ. Therefore, the non-reporter allele is unlikely to be unscathed and is expected to have some mutation. In certain cases, such as when targeting a 3’ end of an ORF, a mutation may not be deleterious. However, a knock-in closer to the 5’ end of an ORF could lead to a heterozygous line with only one functional, reporter, gene copy and another non-functional early stop allele. To avoid such haploinsufficiency, it may be best to select for homozygous knock-in lines using the P2A stitching design we and others have applied to reporter generation. Theoretically, the P2A design allows for homozygous lines to retain “normal” gene function as the protein product is “cleaved” into two functional proteins when the P2A is skipped by the
It is also possible that in the event of a heterozygous line, a unique CRISPR site will be created that would make a correction of that locus possible without disturbing the other allele, but such situations seem unlikely. Rather, caution should be taken when constructing knock-in lines using double strand break inducers, with close attention paid to allele zygosity and a preference for homozygous lines when 2A sites are used. As there may be situations where a heterozygous line is necessary, we would like to propose another method. If two different plasmids were to be supplied for HDR with one reporter knock-in construct and one codon optimized wildtype construct that could not be targeted by the gRNA, a heterozygous clone could be generated by HDR if the cell was to integrate both of the respective sequences into its genome. The codon optimization of the wildtype allele would only need to mask the gRNA binding site, with a simple change of one to three bases likely sufficient. Additionally, to promote integration of both wildtype and reporter sequences, it may be necessary to deliver more of the longer reporter sequence bearing plasmid, as the shorter and more similar codon optimized sequence will likely be preferred for DNA repair. This design should make possible the generation of a true heterozygous cell line with one knock-in allele and one “unscathed” wildtype allele with only slight codon optimization changes.

A recurring theme with using HDR to knock in alleles into the genome of stem cell lines using CRISPR/TALENs has been the low efficiency of this process. Generally, the cells prefer to repair the DNA break using NHEJ. This preference makes it markedly simpler to generate a cell line knock-out than a knock-in. Indeed, a number of groups have worked to improve the rate of HDR in various systems in order to increase the chance of knock-in. However, these systems have so far failed to
work in human stem cells. Other means of improvement of HDR events in stem cells have been to first select for transfected cells via FACS, but this strategy has only yielded an 11% rate of HDR for a simple codon change\textsuperscript{248}. A much smaller rate\textsuperscript{249} is usually seen for relatively larger knock-ins such as GFP because the efficiency of HDR decreases with increasing insert length\textsuperscript{250,251}. However, one group reported a shocking 10-25% HDR efficiency of knocking in a mouse Thy1 gene into human iPSCs when utilizing 2kb or longer homology arms\textsuperscript{251}. It would be interesting to see if this strategy would work in other stem cell lines and for other gene loci or whether it is an isolated event. It also remains possible to increase HDR efficiency through selection of a knock-in event via antibiotic resistance such as puromycin\textsuperscript{252}, but this strategy leaves a “scar” in the genome. Although it is later possible to remove this scar if it is flanked by sites for Flp/FRT and Cre/LoxP recombination systems\textsuperscript{252}, this design adds extra steps that are costly in terms of time and reagents. If a “scar-less” HDR approach had a high efficiency, it would undoubtedly be preferred. As it happens, we have recently developed such an approach, but are still in the early phases of experimentation. Using a plasmid\textsuperscript{202} carrying Cas9-T2A-Puromycin for transient expression and a U6-driven gRNA targeting the 3’ end of the TBP ORF, a housekeeping gene, we have knocked-in a TBP-P2A-GFP into hESCs at efficiencies as high as \sim 90\%. Additionally, we have successfully knocked in a TBP-P2A-tdTomato and SOX2-P2A-GFP reporters into stem cells simultaneously. This approach relies on unique pulsing of puromycin to stem cells two days after electroporation for 24 hours. When performed in this manner, we are selecting for transfected cells, but our observations are rather surprising because we observed a larger increase in HDR than reported by other groups with and without transfection selection\textsuperscript{248,251}. We have named
this technique “puro-HDR” and are currently continuing to explore this method and looking for a mechanism of action. The hypothesis thus far, is that puromycin stalls stem cells in the G2 phase of the cell cycle\textsuperscript{253}, where HDR is more likely to occur\textsuperscript{246}, and perhaps it may also increase the expression of HDR machinery genes as well.

Using puro-HDR, we hope to gain access to many more genetic tools as production of knock-in stem cells lines will no longer be such a difficult and time-consuming endeavor. For example, puro-HDR could be used to knock-in premature stop codons at defined locations, thus selecting for a user-specified knockout cell line. Additionally, we could generate more purification themed stem cell lines, such as BRN3A-P2A-Thy1.1, to affinity purify BRN3A+ RGCs separately from BRN3B+ RGCs using the same stem cell-derived population. We have tested anti-Thy1.1 antibodies for immunopanning of H7 derived RGCs in the past and had observed no binding. Therefore, both Thy1.1 and Thy1.2 could be genetically engineered into human stem cell reporter lines for selection of two separate populations. The MACS system from Miltenyi already provides CD90.1 and CD90.2 microbeads to respectively recognize the two different Thy1 versions. Consequently, we anticipate that in the very near future, we will be able to purify a large collection of various differentiated cell types carrying instructed mutations to model human disease \textit{in vitro} using precisely genome engineered stem cell lines.
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Curriculum Vitae

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- Designed a novel human pluripotent stem cell differentiation protocol for large-scale generation of retinal ganglion cells (RGCs)
  - Used CRISPR-Cas9 technology to genetically engineer an RGC fluorescent reporter hESC line and an alternate version for RGC-specific immunopurification
  - Optimized differentiation parameters to favor RGC genesis and performed small molecule screens to increase RGC yield
  - Characterized differentiation by RT-qPCR and immunohistochemistry
  - Applied fluorescence-activated cell sorting (FACS) to isolate purified RGCs from mixed stem cell culture
  - Imaged RGC activity using calcium imaging and in collaboration studied the electrophysiological properties of the purified RGCs
  - Combined differentiated retinal cultures with nano-fiber matrix scaffolds to study aligned axonal outgrowth
- Determined a minimum set of transcription factors (TFs) necessary for direct reprogramming/transdifferentiation of the human retinal pigment epithelium (RPE) cell line ARPE19 to the RGC phenotype
  - Transferred identified TFs into the human RGC reporter stem cell line to show that RGC yield was increased by TF expression during differentiation
- Developed lentivirus production protocols and engineered new lentivirus expression vectors with variable resistance selection cassettes and fluorescent properties
- Created cell lines for production of neurotrophic factors and secretion of Lucentis: a medically relevant anti-VEGF FaB used in treatment of macular degeneration
- Performed siRNA knockdown in primary mouse RGC cultures for studies of neurite outgrowth and lentivirus shRNA knockdown for studies of stem cell differentiation
- Differentiated stem cells to cardiomyocytes for calcium imaging and a histone post translational modification profiling study

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- Developed and optimized purification of the Fibronectin type III (FN3) domain of the MID1 protein
  - Performed molecular cloning to generate multiple FN3 constructs for bacterial protein expression
  - Purified constructs using affinity chromatography
  - Achieved >95% FN3 purity at suitable concentrations for nuclear magnetic resonance (NMR) analysis
  - Used dynamic light scattering and HSQC to determine that FN3 forms a soluble monodisperse multimeric aggregate that precludes it from structural assignment by NMR
- Assisted with protein purification of other MID1 domains and binding partner proteins

**Scholarships, Fellowships, and Other Funding:**

Maryland Stem Cell Research Foundation TEDCO grant (MSCRFI-0774), contributing author, 5/14-5/16
Visual Neuroscience Training Program Pre-Doctoral Fellowship, National Eye Institute, 12/12-12/14

Oklahoma State Institutional Nominee Academic Scholarship, 8/07-05/10

Wentz Research Scholarship, 08/09-05/10

Niblack Research Scholarship, 08/08-05/09

Howard Hughes Medical Institute CTBS Scholarship, 05/08-08/08

Freshman Research Scholarship, 01/08-05/08

**Awards and Honors:**

04/14  Oral Presentation Award, 2014  JHUSOM
       25th Annual Wilmer Research Meeting

05/10  Paul F. Kruse Award  OSU
       Senior of the Year for Biochemistry and Molecular Biology

05/10  General Honors College Award  OSU

05/08  James E. Webster Award  OSU
       Freshman of the Year Award for Biochemistry

08/07-08/09  OSU Award of Excellence  OSU
             President’s Honor Roll

08/07  Academic Competitiveness Grant  OSU

**Publications:**


Abstracts and Presentations:


