BUILD YOUR OWN RETINA: MODELING RETINOGENESIS AND DISEASE USING HUMAN PLURIPOTENT STEM CELLS

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<td>AMD</td>
<td>Age-related macular degeneration</td>
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<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
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<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>FF</td>
<td>Feeder-free</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<td>hPSCs</td>
<td>Human pluripotent stem cells</td>
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<td>hiPSCs</td>
<td>Human induced pluripotent stem cells</td>
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<td>ICC</td>
<td>Immunocytochemistry</td>
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<td>ipRGC</td>
<td>Intrinsically photosensitive retinal ganglion cells</td>
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<td>IOP</td>
<td>Intraocular pressure</td>
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<td>MEF</td>
<td>Mouse embryonic fibroblasts</td>
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<td>miPS</td>
<td>mRNA-reprogrammed hiPSCs</td>
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<td>NIM</td>
<td>Neural induction medium</td>
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<td>RDM</td>
<td>Retinal differentiation medium</td>
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<td>RPE</td>
<td>Retinal pigmented epithelium</td>
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<td>riPS</td>
<td>Retroviral-reprogrammed hiPSCs</td>
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<td>RGCs</td>
<td>Retinal ganglion cells</td>
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<td>XF</td>
<td>Xeno-free</td>
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ABSTRACT

Sridhar, Akshayalakshmi Ph.D., Purdue University, August 2017. Build Your Own Retina: Modeling Retinogenesis and Disease using Human Pluripotent Stem Cells. Major Professor: Jason S. Meyer.

Human pluripotent stem cells (hPSCs) allow for the unprecedented ability to recapitulate early stages of human development, which are otherwise inaccessible to investigation. This is especially true for one of the earliest events in human development, the establishment of a neuroretinal fate from an unspecified pluripotent population. To test the ability of hPSCs to serve in this capacity, hPSCs were generated using mRNA-reprogramming methods and maintained in xenogeneic-free differentiation conditions. These cells were directed to differentiate using a three-dimensional approach to analyze their ability to successfully recapitulate early events in human development in a temporal and developmentally-appropriate fashion. To do so, hPSCs were first directed to an anterior neural phenotype, which was confirmed by analysis of stage-specific neural transcription factors via immunocytochemistry and quantitative RT-PCR. Next, the cells were directed to an optic vesicle-like stage, where the presumptive retinal cells were identified by the expression of specific transcription factors. Finally, three-dimensional optic vesicle-like retinal organoids were identified, isolated, and further analyzed for the expression of markers associated with some of the differentiated cell types of the neural retina. Upon establishment of hPSC-derived retinal organoids, this system was further utilized to study the neurodegeneration in glaucoma and provide insights into the disease mechanisms. Overall, the results of this study help to demonstrate the suitability of hPSC-differentiation approaches as an effective tool to model retinal development and disease.
1. MODELING RETINAL DEVELOPMENT AND DISEASE USING 3-D RETINAL ORGANOIDS DERIVED FROM HUMAN PLURIPOTENT STEM CELLS

1.1 Introduction

The human retina is a multilayered tissue composed of an intricate network of several types of retinal neurons that function in an integrated manner to convert the incoming light stimulus into an electrical impulse, which will be propagated to the brain to be converted into an image. Consequently, disease or injury affecting retinal neurons that disrupts this visual circuit can result in blindness. Hence, a thorough understanding of the development and functions of the human retina will facilitate the development of successful therapies for retinal diseases. However, studies of the human retina are especially challenging as retinogenesis occurs early in gestation and remains largely inaccessible to investigation [1]. In this regard, human pluripotent stem cells (hPSCs), including human embryonic stem cells [2] and human induced pluripotent stem cells [3–5], provide an unique in vitro model capable of recapitulating the growth and diversification of developing retinal neurons.

hPSCs are self-renewing cells analogous to the blastocyst stage of human development, which possess the exclusive ability to generate all cell types of the body. Therefore, hPSCs can be used to study even the earliest events of retinogenesis and generate limitless numbers of retinal neurons for translational applications [6–15]. While advancements in hPSC-retinal differentiation protocols over the last decade have led to the successful generation of all types of retinal neurons [9, 16–26], these cells have
traditionally been differentiated in a manner that lacked the ability to assemble into a multilayered retinal-like structure. This lack of cellular organization not only affects the ability to faithfully recapitulate the events of retinogenesis as an in vitro model, but may also impact the quality and functionality of retinal cells generated for future translational applications, including disease modeling and cell replacement.

More recently, a fundamental shift in retinal differentiation protocols has developed, which allows for the organization of hPSC-derived retinal neurons into a multilayered retinal-like structure [21,27–29]. These resultant populations, known as retinal organoids, are composed of retinal neurons arranged in a stratified tissue that recapitulates the spatial and temporal patterning of native retinal tissue [21, 28–36]. Thus, such hPSC-derived retinal organoids will likely serve as more effective in vitro models that recapitulate early stages of retinogenesis. Furthermore, these retinal organoids will likely enhance the application of hPSCs for disease modeling and cell replacement. To serve in these capacities, however, refinements in the differentiation of retinal organoids from hPSCs will be needed, with improvements in these protocols likely to be inspired by studies of the developing retina.

1.2 Development and organization of the vertebrate retina

The retina is a complex multilayered tissue that originates from the developing diencephalon and consists of six neuronal cell types that work in a coordinated fashion to perceive and interpret incoming visual information [37,38]. Based on the orientation of these retinal cells, the retina can be broadly classified in three layers: (i) the outer nuclear layer consisting of the photoreceptor cells, including the rods and cones, (ii) the inner nuclear layer consisting of the interneurons, namely bipolar cells, amacrine cells and horizontal cells, and (iii) the ganglion cell layer consisting of the retinal ganglion cells whose axons extend to form the optic nerve [39] (Figure 1.1). In addition to these neuronal cell types, Muller glia are the primary glial cells of the retina, with
Fig. 1.1. Structure of the retina [40]. The retina is located towards the back of the eye and is composed of several different cell types. RPE and photoreceptor cells (rod and cones) occupy the outer layers of the retina, the interneurons (amacrine cells, horizontal cells, and bipolar cells) occupy the central layers while ganglion cells occupy the innermost layers and muller glia transverse the length of retina (not pictured). Image modified from Kimbrel and Lanza et al. 2015.

the nuclei of these cells in the inner nuclear layer and their processes traversing the length of the retina, providing necessary architectural and functional support. Additionally, photoreceptors are also supported and nourished by the retinal pigmented epithelium (RPE), a sheet-like layer of epithelium located behind the photoreceptor layer. These retinal cells are intricately connected through a network of synapses, with connections between the photoreceptors, bipolar cells, and horizontal cells, referred to as the outer plexiform layer. Similarly, the inner plexiform layer represents the dense fibrils between the ganglion cells, bipolar cells, and amacrine cells.

This characteristic order of the retina forms a highly-regulated pathway for visual transduction, which is critical to the functioning of the retina [41]. Briefly, incoming light is focused onto the retina via the lens, where it first interacts with the photoreceptors in the outermost layers of the retinal tissue. These photoreceptors convert the
visual light into an electrical stimulus via the phototransduction pathway, which is then transmitted to the retinal ganglion cells via the interneurons of the retina. Finally, the ganglion cells extend their long axons to exit the eye via optic nerve and synapse with their postsynaptic targets, including the superior colliculus and the lateral geniculate nucleus, thus enabling vision. Overall, the functions of the retina depend on all its components working in a sequential manner to transmit the visual information to the brain. Consequently, any disruption in this visual circuit due to injury or disease to the retinal neurons results in loss of vision or blindness. As such, the use of hPSCs provides a powerful tool for the study of retinal development, as well as disruptions to retinal communications resulting in vision loss [42]. However, modeling the functions of the retina and its pathophysiology requires the differentiation and organization of these cells in a manner which closely recapitulates the in vivo retina, necessitating a thorough understanding of mechanisms associated with retinal development.

Retinal development is determined by the combinatorial actions of growth factors as well as transcription factors, which not only specify retinal cell types but also determine their spatio-temporal location. Retinogenesis begins early in gestation and the first morphological evidence of the retina is seen during neurulation [44]. As the developing neural plate forms the neural tube, optic grooves emerge on either side of the diencephalon. These grooves, now known as optic vesicles, evaginate towards the surface ectoderm, resulting in reciprocal induction between these structures (Figure 1.2). This reciprocal exchange of signals leads to the induction of the retina from the distal optic vesicle and the induction of the lens placode from the ectoderm. Consequently, the proximal optic vesicle is induced by the surrounding mesenchyme to form the retinal pigmented epithelium (RPE). Following specification of the optic vesicle, these cells acquire a multipotent progenitor identity and will subsequently multiply and differentiate into all the major neural cell types of the retina and muller glia. As such, these retinal cells are specified in an evolutionally conserved order, which is
Fig. 1.2. Summary of vertebrate retinogenesis [43]. The eye field is specified from the anterior neural plate, and activation of transcription factors and signaling pathways from the surrounding tissues leads to the patterning of optic vesicle into the neural retinal and retinal pigmented epithelium. Image modified from Parvini et al. 2014.

ddictated by the competence of retinal progenitors, and a combination of exogenous signaling gradients, as well as endogenous transcriptional regulation [45–47]. Based on this model, studies of retinal development in model systems have demonstrated that ganglion cells, horizontal cells and cone photoreceptors are the earliest-born retinal cell types. Amacrine cells are specified slightly later in development, followed by rod photoreceptors while bipolar and muller glia cells are the last cell types to develop in the retina. Retinal development and maturation continues throughout gestation and visual synapses continue to mature after birth. Overall, the specification of the retina from its early diencephalic origins follows a tightly conserved order of events.
Likewise, to properly and reliably direct the differentiation of hPSCs to a retinal fate, a knowledge and application of these developmental signals to cellular differentiation protocols is essential.

1.3 In vitro retinal developmental studies using hPSCs

The development of the human retina is initiated at some of the earliest stages of gestation, making the study of these critical cell fate determination events difficult. Given their pluripotent nature, hPSCs may provide a unique and novel tool for the study of these early developmental events by serving as comprehensive models of the major stages of human retinogenesis, even at stages that would be otherwise inaccessible to investigation. With the resultant retinal cells, the potential then exists for their translational application, including cell replacement approaches, as well as the ability to model and study retinal degenerative diseases in a dish when derived from specific patient sources. In order to serve in this capacity, however, these cells must be directed to differentiate toward a retinal lineage in a step-wise process that faithfully recapitulates the major stages of retinogenesis in vivo [48]. As such, numerous efforts over the last decade have focused on the derivation of retinal cells from hPSCs, often adopting critical principles of developmental biology to guide the differentiation process. Initial work in this field focused upon the differentiation of retinal cells by inhibiting BMP and WNT signaling in the presence of IGF-1 [18,49]. Similarly, other groups have been successful in achieving retinal differentiation through the inhibition of WNT and Nodal signaling [16, 17, 24, 50]. Subsequently, efforts relying upon the default adoption of a rostral neural fate led to the development of discrete retinal progenitor cell populations (Figure 1.3), which would later give rise to retinal neurons in a temporally appropriate manner [20–22,25,26,51].

While these protocols provided the ability to generate all the major cell types of the retina, most of the early focus has favored the generation of RPE and photore-
ceptor cells, as many retinal diseases primarily affect these cells, resulting in their degeneration and subsequent loss of vision. Additionally, RPE and photoreceptors possess unique phenotypic markers and functional properties that enables their ease of identification in vitro, which is often lacking for other cell types of the retina. Among the earliest retinal differentiation studies, RPE was first observed to be spontaneously differentiated from hPSCs in relatively high numbers [52]. These cells were initially identifiable in cultures of differentiating stem cells due to their accumulation of melanin pigment within these cells that could be readily visualized. Further confirmation of RPE differentiation was provided by their characteristic hexagonal shape and upon isolation of these cells, they commonly expressed a full complement of RPE-associated features [21,53–61]. Similarly, photoreceptors were among the first retinal neurons to be identified due to the large number of photoreceptor-specific markers that have been previously identified in retinal development studies [18–20,23,24,49,62]

More recently, some efforts have focused upon the differentiation of retinal ganglion cells from hPSCs. These cells have been somewhat more difficult to definitively identify in differentiating cultures as they lack any truly specific markers to separate them
from other neuronal populations. However, the ability to identify these cells has been facilitated in recent years by following their differentiation through a retinal progenitor intermediary or via the use of fluorescent reporters \[51,63–68\]. Additionally, some studies have demonstrated the ability to derive all the major neuronal cell types of the retina (Figure 1.4), including interneurons, although these cells have not been extensively characterized to date \[18,21,22,24–26,28,69\].

While the above methodologies have been highly successful for the derivation of all of the major types of retinal neurons, this differentiation often occurred as a somewhat heterogeneous population of retinal cells. This differentiation allows for the examination of some features within individual cells, but does not account for the critical interactions between neurons of the retina which are necessary for their proper maturation and function. Furthermore, many disorders of the retina result from the loss of connectivity between cells, making the study of these disorders more difficult in heterogeneously arranged cultures. To overcome these shortcomings, efforts have been directed towards the differentiation of retinal cells from hPSCs in a manner which closely mimics the development and three-dimensional organization of the retina. Initially, studies described the ability of hPSCs to differentiate toward a retinal lineage in a step-wise fashion, yielding three-dimensional structures closely resembling the optic vesicle and optic cup stages of retinogenesis \[21,22,28\]. Subsequently, efforts expanded upon these early results to generate three-dimensional structures termed retinal organoids that were found to effectively recapitulate the spatial and temporal development of the various neuronal cell types of the retina, resulting in a stratified, multi-layered structure \[27,29–35\].

1.4 Applications of retinal organoids for modeling human development

With the goal of effectively recapitulating the complex organization and interplay between the different types of neurons of the retina, studies within the past few years
Fig. 1.4. hPSC-derived cells can be directed to generate all the retinal cell types in a developmentally appropriate manner [21]. Quantitative PCR analysis performed every 10 days of differentiation illustrates the birth-order of retinal neurons (A). hPSCs could be directed to generate all the major cell types of the retina, including retinal ganglion cells (B), amacrine/horizontal cells (C), bipolar cells (D), and photoreceptors (E-H). Image from Meyer et al. 2011.
have described the ability of hPSCs to differentiate toward a retinal lineage in a stepwise fashion [16, 18, 20]. The resultant populations of cells have yielded structures that closely resemble the developing optic cup, with enriched populations of retinal progenitor cells discretely arranged into a cup-like structure (Figure 1.5). Subsequent efforts have expanded upon these early results to generate retinal organoids that effectively recapitulate the spatial and temporal organization of the various neuronal cell types of the retina. As a result, these retinal organoids provide a powerful and novel tool for studies of the earliest stages of human retinal development.

As compared to more traditional methods of retinal differentiation, retinal organoids offer several advantages as an in vitro model of retinogenesis. Importantly, these organoids can self-assemble into discrete three-dimensional structures with major classes of retinal neurons arranged into distinct layers similar to their organization within the retina [21,27–29]. The differentiation of these retinal organoids progresses through all the major stages of retinogenesis, including stages analogous to the eye field, optic vesicle and optic cup, thereby allowing for the ability to visualize some of the earliest events of human retinal development. Similar to embryonic retinogenesis [48], differentiation of resultant cells within retinal organoids have been demonstrated to follow a conserved sequence of events, with early-born cell types such as RGCs among the first retinal neurons to be specified, while later-born cell types such as rod photoreceptors among the last [21,25,28,29,51].

Retinal cells occupy strategic positions within the adult retina, with ganglion cells residing in the innermost layers of the retina, whereas photoreceptor cells closely associate with RPE and form the outermost layers. The spatial arrangement of retinal neurons and their synaptic connections linking them together are critical to their proper function and as such, retinal cells derived from hPSCs should similarly recapitulate this level of organization. While traditional methods of differentiation have allowed for the successful generation of all the major cell types of the retina, these
Fig. 1.5. Morphological identification and isolation of retinal organoids apart from forebrain populations [22]. Retinal organoids possess a distinctive light ring around their periphery, enabling their morphological isolation and enrichment from forebrain populations after a month of differentiation. While both populations express neural genes (\textit{PAX6}, \textit{NeuroD1}, and \textit{FABP7}), expression of retinal genes (\textit{CHX10}, \textit{RAX} and \textit{SIX6}) remained exclusive to retinal organoids and lacked expression of forebrain markers (\textit{SOX1}, \textit{DLX1} and \textit{EMX1}). Image modified from Ohlemacher et al. 2015.
approaches have lacked the ability of retinal cells to assemble into a layered structure. These shortcomings of traditional differentiation approaches have been overcome by the development of retinal organoids, which allow for the maintenance of cell-cell contacts between retinal neurons [21, 27–29]. These organoids formed a pseudostratified epithelium-like structure which allow the retinal cells to mature in both a temporal and spatial fashion, with ganglion cells specified in basal regions of the organoids and photoreceptors in apical regions.

The three-dimensional nature of organoids also likely aids in the functional maturation of retinal neurons, which has been largely limited in retinal cells derived using traditional differentiation methods. While they express a variety of features associated with all of the major cell types of the retina, they lacked the structural and functional differentiation typically associated with more mature retinal neurons. The use of three-dimensional retinal organoids allows for the acquisition of more advanced features of differentiation within these cells, presumably due to their ability to interact and self-organize with neighboring cells. Further refinements of these organoid cultures have also involved the addition of external signaling molecules in long-term cultures to further guide their differentiation [29–32, 34, 35]. This has been particularly true for photoreceptors, which have been the most extensively studied cell type derived within retinal organoids. The experimental manipulation of critical signaling pathways within retinal organoids has led to refinements in photoreceptor differentiation, including accelerated differentiation and increased expression of phototransduction proteins. Photoreceptors derived in this fashion exhibited characteristic bulb-like structures at their tips, demonstrated membranous disc-like structures in regions resembling outer segments, and occasionally displayed responses to light stimuli [29, 35].
1.5 Application of hPSC-derived cells for disease modeling

Beyond the applications of hPSCs for modeling retinal development, these cells also serve as powerful and unique platforms for the study of human retinal degenerative diseases. Due to the degeneration of specific populations of retinal neurons, these diseases result in loss of vision and eventual blindness. Retinal degenerative diseases can be readily classified into those diseases that affect cells of the outer retina or those affecting the inner retina, most notably age-related macular degeneration and glaucoma, respectively [70, 71]. Traditionally, the ability to study the progression of these disease states has been limited to animal models of the disease. While these animal models have led to significant advances in our understanding of retinal disease progression [72–76], important differences exist between the retinas of animal models and humans. Furthermore, studies in humans have been largely limited to postmortem retinal tissue or to retinal imaging approaches that lack the resolution to examine individual cells. While these studies have been informative about the end-result of disease pathology, the approach necessarily limits the ability to better understand disease progression within individual cells.

In order to overcome these shortcomings for studies of retinal degenerative diseases, recent research has focused on the use of hPSCs to model and understand disease progression. When generated from patients with a known genetic basis for retinal degeneration, hPSCs provide an renewable supply of cells for the derivation of the affected cell type, and can thereby serve as powerful tools to study the disease phenotype [77–79]. Over the last several years, studies have utilized hPSCs for studies of degenerative diseases of the retina, with a particular focus on those diseases that affect RPE and photoreceptors [11, 15, 21, 53, 55, 58, 59, 80–86]. These cells are often affected in retinal degenerative diseases such as age-related macular degeneration, and the derivation of these cells has been extensively characterized through hPSC retinal differentiation protocols. Such approaches have helped to demonstrate the improper
function and/or reduced survival of RPE and photoreceptors in patient-derived cells, thereby providing insight into potential mechanisms underlying the loss of these retinal cell types [11, 21, 59, 69, 87, 88]. Patient-derived hPSCs have furthermore been utilized to identify novel genetic variants underlying retinal degeneration, highlighting the potential to development mutation-specific therapies [78, 89, 90].

While diseases affecting cells of the outer retina have been extensively studied with hPSCs, studies related to diseases affecting inner retinal neurons have been limited. Of the diseases affecting inner retinal neurons, the most common is glaucoma with a current incidence of greater than 60 million people worldwide [91, 92]. Glaucoma results in the degeneration of retinal ganglion cells (RGCs), leading to a decreased connectivity between the eye and the brain and subsequent loss of vision. The ability to derive RGCs from hPSCs has been a more recent area of investigation [51, 65–68, 93], which now allows for the application of these cells for studies of retinal degenerative diseases affecting the inner retina. Recently, efforts have focused on the use of hPSCs from patients with genetic determinants of degenerative diseases that directly affect the RGCs. Interestingly, upon the differentiation of these cells, RGCs from patient sources exhibited increased apoptosis, thereby allowing for subsequent studies of disease mechanisms leading to degeneration of RGCs [51, 94].

While traditional retinal differentiation protocols have been highly successful in modeling certain features of some retinal degenerative diseases, the resultant retinal cells differentiate in a manner that lacks any three-dimensional organization that mimics how cells are arranged into retinal tissue. As these hPSC-derived retinal cells have not accurately recapitulated the stratified organization of the retina, their ability to accurately recapitulate many features of retinal degenerative diseases has often been restricted. With the three-dimensional differentiation of retinal organoids, hPSC-derived retinal cells closely associate with each other to form a pseudostratified epithelium, resembling the developing retina [21, 27–29, 35]. These organoids may serve
as an improved model for studies of retinal disease modeling, allowing for the interaction between different cell types and therefore providing the ability to assess the effects of degeneration on the entire tissue [51, 78, 95]. While such approaches have not been demonstrated yet with retinal organoids, the concept has been effectively described in organoid cultures in other neural systems. As an example, hPSC-derived organoids have been particularly successful for modeling some of the effects of cerebral diseases, such as microcephaly and lissencephaly [96, 97]. In the near future, it is likely that retinal organoids will be applied for the study of retinal degenerative diseases. As recent studies have demonstrated the successful organization and maturation of photoreceptors within these organoids, [29, 35], these approaches will most likely be applied for diseases primarily affecting the outer retina. The most appropriate diseases for modeling with hPSC-derived organoids will likely possess a genetic basis versus an idiopathic basis for disease. Additionally, further improvements to retinal organoids will likely be necessary to be able to apply them to a wide variety of retinal degenerative diseases. As hPSC-derived retinal organoids are yet to demonstrate a macula-like region or a functioning RPE layer, they may be better suited to model diseases that affect peripheral photoreceptors such as retinitis pigmentosa. Further improvements in the differentiation methods to also include the characterization and maturation of inner retinal neurons will enable the study of diseases affecting ganglion cells with retinal organoids.

1.6 Application of hPSCs for drug screening purposes

When derived from individual patient populations, particularly those with a known genetic basis underlying retinal disease, hPSCs possess the ability to recreate certain features of the disease phenotype and model the degeneration associated with retinal diseases. With the resulting data accumulated from such studies, these cells can then be utilized for the development of therapeutic approaches for retinal degenerative diseases [79, 85, 98–100]. Following the directed differentiation of patient-derived hPSCs
to a retinal fate, drug screening efforts can be targeted to an affected retinal cell type, providing a platform for assessing the ability of candidate compounds to rescue the disease phenotype.

The use of patient-derived hPSCs for drug screening has been particularly successful for degenerative diseases that affect the outer retina, whose cells have been routinely derived and extensively characterized from hPSCs [6, 11, 14, 21, 69, 84, 95, 101–106]. Photoreceptors and RPE are the most common cell types affected in many retinal degenerative diseases such as age-related macular degeneration (AMD), where the loss of photoreceptors combined with dysfunctions in RPE lead to loss of vision. As patient-derived RPE has been shown to recapitulate some of the hallmark features of AMD, including elevated expression of inflammatory factors and defective oxidative stress responses, recent studies have utilized hPSC-derived RPE as a platform for the screening of candidate drugs to assess the ability to improve their survival [33, 59, 107, 108]. The results of these studies have enabled the identification of select compounds such as nicotinamide and curcumin as potential neuroprotective agents that can alleviate RPE degeneration [104, 109]. Similarly, hPSC-derived retinal cells have also been utilized for drug screening purposes as a means to alleviate photoreceptor loss due to retinitis pigmentosa, with results indicating that hPSC-derived photoreceptors were able to recapitulate the disease phenotype and upregulate markers of oxidative stress, lipid oxidation and apoptosis [11, 90, 102, 103, 106]. Correspondingly, treatment of the degenerating rod photoreceptors with antioxidant vitamins effectively increased photoreceptor survival.

While hPSC differentiation strategies initially emphasized the cells of the outer retina, recent refinements in differentiation protocols have enabled the stepwise differentiation and identification of inner retinal neurons, particularly RGCs [51, 63, 65–68, 94]. As RGCs serve as the critical connection between the eye and the brain to transmit visual information, their degeneration often leads to the onset of diseases known as
Fig. 1.6. Modeling RGC degeneration with patient-derived hPSCs [51]. hPSCs were generated from a patient with a E50K mutation in the Optineurin gene (OPTN) associated with glaucoma and were directed to a retinal phenotype (A-B) and subsequently to a retinal ganglion cell fate (C-D). While pluripotent control and patient-hPSCs exhibit no significant differences in expression of apoptosis markers (E-G), OPTN-RGCs demonstrated significantly elevated caspase expression after 70 days of differentiation (H-J). Treatment with neuroprotective factors lead to significant reduction in markers of apoptosis (K-M). Significant differences indicated as ( * = p < 0.05, *** = p < 0.005 ). Scale bars equal 400µm in A, 100µm in B, 50µm in C-F, and 25µm in H-L.
optic neuropathies, resulting in vision loss and eventual blindness. RGCs differentiated from hPSCs, particularly when derived from patient-specific sources, allows for the ability to screen new drug compounds and develop personalized treatment profiles for optic neuropathies [51,94]. As a proof of principle, recent studies have successfully demonstrated the ability to faithfully recapitulate some of the degenerative processes associated with optic neuropathies in hPSC-derived RGCs, with subsequent drug screening approaches enabling the identification of neurotrophic factors capable of rescuing RGC degeneration [51](Figure 1.6).

While a number of studies have successfully demonstrated the ability to screen compounds for their neuroprotective effects on hPSC-derived retinal cells [21,59,87], these approaches have focused on isolated cells lacking any three-dimensional organization reminiscent of retinal tissue. With the advent of retinal organoids, hPSCs can be directed to differentiate in a manner that recapitulates the architecture, spatial connectivity and functioning of the retina, and may therefore be better suited for drug screening purposes. Given the more detailed demonstration to date of photoreceptor differentiation and organization in the outer layers of retinal organoids, these cells are likely better suited for drug screening applications for photoreceptor diseases. In contrast to outer retinal diseases, retinal organoids can also be used to test and develop therapies for inner retinal neurons such as RGCs, which are primarily affected in optic neuropathies.

1.7 hPSCs-retinal cells for future cell replacements

While early stages of retinal degenerative diseases may be effectively studied with hPSCs, and subsequently drug screening approaches may aid in the neuroprotection of these degenerating cells, the irreversible loss of retinal neurons in later stages renders such measures ineffective, resulting in severe vision loss and blindness. In such cases, attempts to replace degenerated cells through transplants of healthy retinal cells con-
stitutes the only remaining effective option to restore some visual function \[110,111\]. The transplantation of cells into the retina represents a more feasible option for cell replacement when compared to other cells of the nervous system, as the relative ease of accessibility of the retina and its reduced immunological response will likely facilitate cell replacement therapies \[112,113\]. To aid in this goal, hPSCs can serve as a renewable source of stem cells for the differentiation of retinal cells for a variety of translational approaches to retinal repair. Transplants of hPSC-derived retinal cells can assist in neuroprotection, particularly at earlier stages of the disease process, and can lead to potential delay in disease progression. At later stages of the degenerative process, hPSC-derived retinal cells can serve as a source for repopulation of the retina following the loss of host neurons.

Several studies have examined the use of hPSC-derived retinal cells for cell replacement in diseases that affect photoreceptors, with the goal to replace the degenerating neurons with their functional equivalents \[49,78,114–116\]. Initial studies focused on transplantation of retinal stem cells into animal models, which could integrate into many layers of the retina and exhibit neuronal morphologies \[117–119\]. However, these cells were often limited in number and their ability to be expanded, and rarely exhibited any ability to give rise to photoreceptor cells. As an alternative, more recent efforts have focused upon the ability of hPSC-derived photoreceptor cells for cell replacement. Upon transplantation, several groups have demonstrated the ability of these cells to integrate into the host retina and form connections with other retinal neurons, in some cases leading to improved visual function and restoration of light sensitivity \[49,120,121\]. Further investigations into the transplantation of hPSC-derived photoreceptors have indicated the ability of anti-apoptotic factors or the use of immunodeficient mouse models to improve survival of hPSC-derived photoreceptors \[116,122\].
As the RPE provides essential support for photoreceptors, similar approaches for cell replacement have also been developed for RPE loss in retinal degenerative diseases, often associated with the subsequent loss of photoreceptors and disruption of the visual pathway. hPSC-derived RPE has been utilized in the development of cell replacement strategies for diseases such as age-related macular degeneration [15,82,104,105,107]. In this capacity, the transplantation of RPE cells has been accomplished by either the injection of RPE cells as a cell suspension or as a sheet-like layer of RPE [123–129]. The latter approach may offer numerous advantages, as the cells retain their polarization and ensures the cells are arranged in a discrete monolayer, allowing the transplanted RPE to better integrate within the host retina. Such approaches have been successful in rescuing some degeneration of the photoreceptors due to transplanted RPE. The success of these approaches has led to the utilization of hPSC-derived RPE in clinical trials for AMD and Stargardt’s disease, where transplanted cells were shown to improve visual acuity in patients, illustrating the ability of hPSCs to rescue visual defects in retinal degenerative diseases [14,130].

Many of the cell replacement strategies developed to date have focused on the transplantation of RPE and/or photoreceptors due to their ease of differentiation and more limited need of these cells to extend neurites to form synaptic connections, which will likely make replacement efforts easier. However, the development of replacement strategies for inner retinal neurons such as RGCs are more complicated, largely due to their more elaborate nature and need to extend long distance projections in order to form synaptic connections [131–133]. As such, strategies to combat RGC degeneration have traditionally focused upon early stages of the disease process where neuroprotection is feasible [51,94], with the eventual goal of not only aiding in survival of RGCs but also the regrowth of axons toward the reestablishment of synaptic connections. Similar efforts have not been widely adopted yet for hPSC-derived RGCs, although early studies have demonstrated the ability of hPSC-derived RGCs to survive following transplantation into the vitreous chamber [64]. Further studies
into the use of hPSC-derived RGCs are certainly warranted, as several recent studies have demonstrated the differentiation and enrichment of RGCs from hPSCs in vitro.

While efforts for cell replacement to date have often focused on the transplantation of a single type of retinal neuron, the restoration of vision will require the proper integration of these cells into the retinal circuitry. At particularly late stages of the disease process, other retinal neurons are often damaged and lost, leading to the need to replace multiple types of cells and reestablish their connections as well. Retinal organoids represent an exciting option for cell replacement at these late stages of retinal degeneration, as these organoids possess the relevant retinal cells pre-assembled into a stratified structure, and can serve as “mini-retinas” for replacement of retinal tissue in degenerative diseases [21, 28–30, 35, 36]. Early attempts at these strategies have recently been demonstrated in mouse models of retinitis pigmentosa. Following the transplantation of retinal organoids, these cells retained their three-dimensional architecture and formed rudimentary synaptic connections with host bipolar cells [123]. Similar experiments have also been conducted in non-human primates, with the transplantation of hPSC-derived retinal organoids resulting in increased visual acuity [134].

1.8 Conclusions and future directions

Overall, research over the past several years has established hPSCs as a powerful tool for studying some of the earliest stages of human development that would otherwise remain inaccessible to investigation [17, 18, 20, 22, 26]. Related to this ability, many studies during this period have demonstrated the use of hPSCs as an exceptional model for studies of human retinal development, facilitating the establishment of efficient differentiation protocols to generate all major cell types of the retina, including photoreceptors, RPE and retinal ganglion cells [19, 21, 29, 35, 49, 51, 53, 60, 62, 65, 68, 81, 88, 135]. These resultant hPSC-derived retinal cells have assisted in modeling retinal degener-
ative diseases, especially when generated from patients with a genetic susceptibility to the disease. For this purpose, patient-derived hPSCs have helped in understanding disease progression and mechanisms, and have subsequently enabled the identification of candidate neuroprotective factors to combat the degeneration of retinal neurons [11, 21, 51, 69, 78, 87, 89, 94, 95, 102, 104, 108, 128]. However, these measures have been unsuccessful at penultimate stages of the disease process, where the loss of multiple types of retinal neurons severely impacts retinal interconnectivity, leading to a lack of proper synaptic communication between these cells and a resulting severe loss of vision. Upon the loss of retinal neurons in this manner, hPSC-derived retinal cells can also serve as a source for cell replacement, where transplants of hPSC-derived retinal neurons have been shown to integrate within the host retina, form synaptic connections and well as enable some rescue from disease symptoms. Such strategies have been extensively applied for the rescue of RPE and photoreceptor degeneration [6, 10, 56, 78, 116, 121, 123, 134, 136, 137], leading to the development of hPSCs-derived retinal cells for clinical trials in AMD and Stargardt’s disease [14, 130].

While tremendous progress has been made in the differentiation of retinal neurons from hPSCs [16, 18–20, 26, 51, 67, 80, 88, 138, 139], these cells often fail to fully differentiate into functionally relevant phenotypes which would better mimic the structure and functionality of the retina. Therefore, recent advances have led to the development of a three-dimensional approach to retinal differentiation, where hPSCs are directed to yield discrete populations closely analogous to the developing optic cup and eventually giving rise to a pseudostratified structure resembling the retina [21, 22, 27–30]. With these advances, retinal organoids follow predicted stages of retinal development, and have led to enhanced differentiation and maturation of photoreceptors, facilitating the application of these approaches for studies of the retina in both normal and diseased states.
As opposed to retinal cells derived from traditional differentiation methods, patient-derived organoids may be better suited for assessing the effects of disease-related neurodegeneration on specific retinal cell types, as well as their interactions with each other. Currently, retinal organoids are likely better suited for studies of photoreceptor diseases, as photoreceptor development and maturation has been extensively characterized in retinal organoids, leading to rod-dominant retinal domains similar to peripheral regions of the retina [28,29,32,34–36]. Therefore, degenerative diseases like retinitis pigmentosa, which primarily affects these regions, can be most effectively modeled with retinal organoids, with the goal to develop neuroprotective strategies to combat photoreceptor degeneration. Moreover, future efforts to characterize inner retinal neurons within retinal organoids will help to model and develop potential therapies to combat RGC degeneration in optic neuropathies. In addition to studies of retinal development and disease, the most exciting feature of retinal organoids may be their ability to serve as a replacement for retinal tissue in severely degenerated retinas. Retinal organoids innately organize into a pre-assembled and interconnected structure composed of multiple retinal neurons, and may facilitate integration and replacement of cell types within the degenerated retina.
2. NON-XENOGENEIC DIFFERENTIATION OF HUMAN PLURIPOTENT STEM CELLS INTO A RETINAL LINEAGE

2.1 Introduction

Human induced pluripotent stem cells (hiPSCs) have been implicated to hold great potential for regenerative medicine because of their ability to generate any cell type of the body as well as their unique ability to generate patient-specific cell populations. These cells maintain the entire unique set of genomic information for each individual patient, representing a great opportunity for the development of personalized treatment profiles for a wide spectrum of diseases. [77, 83, 140–143]. However, significant issues still need to be addressed before their full potential is realized, as graft rejection and zoonosis constitute two major issues resulting from the use of animal products or other undefined components during routine culture of these cells. These risks must be minimized or eliminated before effective cell replacement therapy can be realized [50,144]. Hence, a necessary step in this field of research is the development of non-xenogeneic differentiated progeny derived from hiPSCs which could then be successfully used for translational research and regenerative medicine.

Traditionally, the use of human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and hiPSCs, for retinal applications has relied upon their growth on a layer of mitotically inactivated mouse embryonic fibroblasts (MEFs) in the presence of medium containing Fetal Bovine Serum (FBS) or Knock Out-Serum Replacement (KOSR) using animal components [16, 18, 19, 23, 54, 62]. Additional efforts have been made to eliminate the use of undefined growth conditions through the use of feeder-free systems utilizing chemically defined media [145–149]. However,
such approaches often rely upon defined or semi-defined animal constituents in the medium, as well as the growth of cells upon a matrigel substrate. More recent efforts have focused on the growth of hPSCs under non-xenogeneic conditions, along with the differentiation to limited cellular lineages [150–154]. However, the successful growth and differentiation of hPSCs to a retinal lineage has been largely unexplored. Thus, for future retinal applications, a need exists to establish conditions for the growth and differentiation of hPSCs in the absence of xenogeneic materials.

The use of MEF and feeder-free systems for the growth of hiPSCs has been reviewed extensively in literature [16, 20, 23, 88, 155], allowing for their use as controls for our experiments. We have previously demonstrated the derivation of a variety of retinal cells from hPSCs including photoreceptors, retinal ganglion cells and retinal pigmented epithelium (RPE) using a stepwise differentiation protocol [20, 21]. In the current study, we have adapted this procedure to demonstrate the growth and differentiation of hiPSCs to a retinal phenotype in the absence of xenogeneic materials. Care was taken to ensure all ancillary components used in the culturing of hiPSCs were free of animal origin. Here, we present the first xeno-free approach to the growth and differentiation of hiPSCs into retinal cells that closely resembles previously established methods for derivation of retinal cells. These results represent an important step in the advancement of hiPSCs toward translational purposes.

2.2 Methods

2.2.1 Maintenance of undifferentiated colonies

The IMR90-4 line of hiPSCs was maintained in the undifferentiated state under three different conditions; MEF, feeder-free (FF) and xeno-free (XF). For the MEF system, hiPSCs were grown on plates of mitotically-inactivated MEF, with the supplementation of hiPSC medium (DMEM-F12 with 20% Knockout Serum, Cat# 18028), 0.1 mM $\beta$-mercaptoethanol, 1 mM L-glutamine, MEM Non-Essential Amino Acids,
and 4 ng/ml FGF2) on a daily basis. The feeder-free system comprised of Matrigel coating of plates (BD Biosciences, Cat# 354277) and the use of mTESR1 medium (Stemcell Technologies, Cat# 05870) for maintenance of cells in an undifferentiated state. For the xeno-free system, cells were grown on Synthemax plates (Corning, Cat# 3877XX1) in Nutristem medium (Stemgent, Cat# 01-0005).

Cells were passaged approximately every four to five days, at 70-80% confluency. Colonies containing clearly visible differentiated cells were manually removed before passaging. The remaining colonies were lifted off the plate enzymatically by treatment with dispase (2mg/ml) followed by three washes with DMEM-F12. Colonies were then broken up into smaller clusters by manual trituration and were replated at a ratio of 1:6.

2.2.2 Differentiation of hiPSCs

Colonies of undifferentiated hiPSCs grown in FF and MEF conditions were directed to differentiate via the formation of embryoid bodies (EBs) through treatment with dispase, as described previously [20,21]. For XF samples, colonies were mechanically isolated with a cell scraper instead of dispase to gently remove the colonies from the plates. Cells grown in the MEF system were initially grown in EB medium (hiPSC medium minus FGF2) for four days and then transferred to neural induction medium (NIM; DMEM-F12 (1:1), 1% N2 supplement, MEM Non-Essential Amino Acids, 2µg/ml Heparin). Cells grown under feeder-free and xeno-free conditions were slowly transitioned into NIM by transferring the EBs to a 3:1 ratio of mTESR1/Nutristem:NIM on day 0, 1:1 on day 1, 1:3 on day 2 followed by transfer to complete NIM at day 3 of differentiation. To ensure non-xenogeneic growth of cells, NIM lacking heparin was used in xeno-free samples.
At day 7 of differentiation, the cells grown under MEF and feeder-free systems were plated on 6 well plates coated with laminin (20µg/ml) while Synthemax plates were used for non-xenogeneic cultures. Cells acquired advanced neural rosette morphology after 17 days of differentiation followed by their transfer to retinal differentiation medium (RDM; DMEM-F12 (3:1), 2% B27 supplement (non-xenogenic B27 (Life Technologies, Cat# A11576SA) was used for XF cultures) and 1% penicillin-streptomycin. Retinal neurospheres identified by a bright ring appearance around the periphery were manually picked as previously established (Meyer et al., 2009). Neurospheres were fed every 2-3 days and were maintained in suspension up to 60 days of differentiation. For RPE differentiation, cells were kept adherent at day 17 of differentiation and were fed with RDM every 3-4 days until approximately 60 days of differentiation.

RPE were cultured as previously described [21]. Briefly, pigmented patches were micro-dissected manually and re-plated on poly-ornithine/laminin coated coverslips. RPE cells were expanded in RDM supplemented with FGF2 (20ng/ml), EGF (20ng/ml) and heparin (2µg/ml) for one week. Mitogens were then removed for 2 weeks and cells were stained as described above.

2.2.3 Immunocytochemistry (ICC)

Samples were collected at specified timepoints of differentiation and were plated onto coverslips coated with poly-ornithine and laminin (20µg/ml) for FF and MEF samples, and poly-ornithine and Synthemax (0.025 mg/ml) for XF samples. They were fixed with 4% paraformaldehyde for 30 minutes followed by three washes with PBS for five minutes each. Cells were treated with 0.2% Triton X-100 for ten minutes followed by blocking for an hour in 10% donkey serum. Primary antibody was added at the recommended dilution in 0.1% Triton-X and 5% donkey serum and incubated overnight at 4°C. Primary antibody was then removed and cells were washed 3X
Table 2.1.
List of antibodies used for Immunocytochemistry (A-M)

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with PBS followed by blocking with 10% donkey serum. The secondary antibody was diluted along with DAPI in 0.1% Triton-X and 5% donkey serum for one hour. Samples were washed 3X with PBS and mounted on slides for fluorescence microscopy. A complete list of antibodies used for immunocytochemistry can be found in tables 2.1 and 2.2.
Table 2.2.
List of antibodies used for Immunocytochemistry (N-Z)

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<td>1:100</td>
<td>Stemgent</td>
</tr>
<tr>
<td>TRA-1-60 (live cell)</td>
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<td>1:100</td>
<td>Stemgent</td>
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<tr>
<td>TRA-1-81</td>
<td>Mouse</td>
<td>1:100</td>
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<tr>
<td>ZO-1</td>
<td>Rabbit</td>
<td>1:100</td>
<td>Invitrogen</td>
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</tbody>
</table>
2.2.4 RT-PCR

Cells were collected at specified time points of differentiation and RNA was extracted using PicoPure RNA Isolation Kit (Applied Biosystems) or RNeasy RNA isolation kit (Qiagen). cDNA was generated using the iScript cDNA Synthesis Kit (Bio-Rad) or SuperScript III First-Strand Synthesis System (Invitrogen). PCR amplification was performed using GoTaq qPCR Master Mix (Promega). PCR experiments were run for 30 cycles followed by analysis of PCR products on 2% agarose gels. A complete list of primers used for PCR can be found in tables 2.3 and 2.4.

2.2.5 Data quantification

Images were quantified using a two-fold approach. First, positive aggregates were identified by nuclear co-localization of antibody and DAPI signal and ImageJ software was used to determine the percentage of positive cellular aggregates. Additionally, each positive cellular aggregate was assessed to determine the percentage of positive cells as compared to total number of DAPI-positive nuclei. A minimum of three images were taken of positive aggregates across a minimum of five samples. Image J software was used to quantify the number of nuclei and antibody of the images. Percentages of positive aggregates as well as positive cell staining were statistically evaluated using unpaired T-test or ANOVA analyses to determine significant differences across experimental growth conditions.

2.3 Results

2.3.1 Maintenance of pluripotency under MEF, feeder-free, and xeno-free conditions

As a prerequisite to xeno-free differentiation of hiPSCs, the ability to effectively expand hiPSCs and maintain pluripotency in the absence of feeder layers and animal derived-products must be established. Thus, the first experiments were designed to
<table>
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<th>Reverse Sequence</th>
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<td>α-FETOPROTEIN</td>
<td>AGA ACC TGT CAC AAG CTG TG</td>
<td>GAC AGC AAG CTG AGG ATG TC</td>
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<tr>
<td>BRACHYURY</td>
<td>ACC CAG TTC ATA GCG GTG AC</td>
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<tr>
<td>β-III TUBULIN</td>
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<td>BEST1</td>
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<td>BRN3</td>
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<td>CHX10</td>
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<td>CRX</td>
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<td>CTCF</td>
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<td>ISLET1</td>
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<td>KLF4</td>
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<td>TGC TCG GTC GCA TTT TTG GCA C</td>
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## Table 2.4.
Primer sequences used for RT-PCR (L to Z)

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<th>Reverse Sequence</th>
<th>Size (bp)</th>
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<td>LHX2</td>
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<td>MITF</td>
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<td>TCT GCT GGA GGC TGA GGT AT</td>
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<td>NR2E1</td>
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<td>OTX2</td>
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<tr>
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<tr>
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<td>GAA TCT CGA AAT CTC AGC CC</td>
<td>CTT CAC TAA TTT GCT CAG GAC</td>
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<td>RPE65</td>
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<td>SIX3</td>
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<td>SIX6</td>
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<td>CTC TGG ACC AAA CTG TGG CG</td>
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<tr>
<td>SOX2</td>
<td>CCC CCG GCG GCA ATA GCA</td>
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<tr>
<td>TBX2</td>
<td>TGG ACA GTT CAC CAT GGG CCC T</td>
<td>GCC TCC GAA AGT GGG CAT TGG A</td>
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Fig. 2.1. Maintenance of pluripotency under xeno-free, feeder-free, and MEF growth conditions. Colonies grown under xeno-free, feeder-free and MEF system of development exhibited similar morphological features when viewed under bright field microscope (A). Uniform and homogeneous expression of pluripotency-associated factors like OCT-4, NANOG and SOX2 was observed in the undifferentiated colonies, irrespective of the system they were grown in (B-D). Similar expression of cell surface markers like SSEA-4, TRA-1-61 and TRA-1-81 was observed in the xeno-free cells when compared to feeder-free and MEF systems, further confirming the pluripotency of these cells (E-G). RT-PCR analysis confirmed the expression of pluripotency genes in hiPSCs maintained in all conditions, as well as the relative absence of markers of differentiation (H).
analyze and compare pluripotency characteristics in hiPSCs cultures under these three conditions. After a minimum of five passages in either MEF, feeder-free or xeno-free systems, colonies of hiPSCs exhibited a uniform appearance without marked differences in the morphologies of the colonies under different culture conditions (Figure 2.1 A). Under all three conditions, immunocytochemistry results confirmed the expression of key pluripotency associated factors in colonies of hiPSCs including OCT4, SOX2, NANOG, TRA-1-60, TRA-1-81 and SSEA-4 (Figure 2.1 B-G). Maintenance of the pluripotent state was further confirmed through RT-PCR analysis, in which key pluripotency genes were found to be expressed under all conditions (Figure 2.1 H). In addition to the expression of characteristic pluripotency genes, colonies of hiPSCs grown under each culture condition also largely lacked the expression of differentiation markers including α-FETOPROTEIN, PAX6 and BRACHYURY, further confirming their undifferentiated state.

2.3.2 Specification of neural and retinal progenitor cells

Prior to the specification of mature retinal cell types, hiPSCs undergo a series of differentiation events analogous to the major stages of retinal development including the primitive eye field, optic vesicle and optic cup [20,21]. Following modifications to previously established protocols, hiPSCs initially acquired features of the primitive anterior neuroepithelium, including the eye field. Immunocytochemistry experiments illustrated the expression of transcription factors SOX1, SOX2, PAX6, OTX2, LHX2, and SIX6 (Figure 2.2 A-F), which were collectively indicative of the acquisition of an anterior neural phenotype. Importantly, inappropriate regional and temporal gene expression was not observed within these cells, confirmed by lack of expression of markers associated with other germ layers (BRACHYURY and AFP; Figure 2.2 G) and posterior markers of the midbrain and hindbrain (EN-1, HOXB4; Figure 2.2 G).
Fig. 2.2. Primitive retinal specification of human induced pluripotent stem cells (hiPSCs) under xeno-free conditions. Within the first 10 days of differentiation, hiPSCs acquired features of the primitive anterior neuroepithelium under all growth conditions (A-D). The near uniform expression of SOX1, SOX2 and PAX6 indicated the acquisition of a primitive neural fate from hiPSCs. hiPSCs also expressed retinal-associated genes including OTX2, LHX2, and SIX6 (E, F). RT-PCR analysis highlighted similar patterns of gene expression across all growth conditions at this time point. Importantly, in addition to the expression of early neuroretinal genes, the regional and temporal specificity of gene expression was confirmed through the absence of genes including EN-1, HOXB4, BRACHYURY, and AFP (G). Quantification of immunocytochemistry experiments revealed comparable percentages of cell aggregates that expressed indicated transcription factors (H), as well as equivalent percentages of cells within immunopositive aggregates expressing each transcription factor (I).
RT-PCR analysis also confirmed the establishment of a retinal identity within the anterior neural population, demonstrating the expression of eye field transcription factors (EFTFs) [156], including PAX6, RAX, SIX3, SIX6, and LHX2 (Figure 2.2 G). To ensure that possible differences among growth conditions were not due to the lack of heparin in the non-xenogeneic culture condition, a comparative analysis was performed which demonstrated a potentially superfluous inclusion of heparin in traditional (MEF and FF) neural differentiation protocols (Figure 2.3) [20, 21, 157, 158]. Importantly, the expression patterns of all transcription factors at this stage were consistent under all three conditions tested, as confirmed by RT-PCR data (Figure 2.2 G) and manual counts (Fig 2.2 H-I), illustrating the potential to derive retinal cell types under non-xenogeneic conditions (Figure 2.2 G).

Following the acquisition of a primitive eye-field phenotype, hiPSCs were directed to differentiate through subsequent stages of retinal development, including those analogous to the optic vesicle and optic cup. We have previously demonstrated the ability to identify and isolate two morphologically distinct populations of cells in cultures of differentiating hiPSCs within the first twenty days of differentiation with characteristics analogous to retinal and forebrain progenitor cells, respectively [21]. Neurospheres previously demonstrated to acquire a retinal fate possessed a phase-bright ring around the periphery (Figure 2.4 A) were manually isolated and analyzed for the expression of retinal progenitor markers.

Immunocytochemistry and RT-PCR analyses demonstrated the expression of these transcription factors in xeno-free, feeder-free and MEF conditions. Global analyses revealed the expression of a full complement of retinal progenitor cell-associated transcription factors including CHX10 (Figure 2.4 B), MITF and TBX2 (Figure 2.4 F). The expression of retinal and neural transcription factors such as PAX6, LHX2, SIX6 (Figure 2.4 C-E) and RAX (Figure 2.4 F) were also maintained in these neurospheres. Additionally, it is important to note the coexpression of many of these transcription
Fig. 2.3. Heparin-independent anterior neural specification of hiPSCs. hiPSCs grown in the presence (+heparin) or absence (-heparin) of heparin under xeno-free conditions were analyzed after 10 days of differentiation to assess the necessity of heparin for neural specification. Immunocytochemistry experiments demonstrated no apparent differences in the ability of hiPSCs to express the anterior neural-associated transcription factors SOX1, SOX2, PAX6, OTX2, LHX2 and SIX6, either in the presence or absence of heparin (A-F). Quantification of these immunocytochemistry experiments demonstrated that these transcription factors were uniformly expressed in nearly all cellular aggregates (G). Within those aggregates that expressed these transcription factors, greater than 95% of all cells were immunopositive for these proteins (H). No statistically significant differences were observed between +heparin and -heparin populations of cells.
Fig. 2.4. Derivation of definitive retinal progenitors from hiPSCs under xeno-free conditions. Under each of the three growth conditions, retinal progenitor spheres can be isolated under brightfield microscopy using morphological cues (A). The retinal spheres were identified by a light outer ring around the periphery, a morphological feature absent in non-retinal spheres. Retinal progenitor cells expressed characteristic markers such as CHX10, PAX6, LHX2 and SIX6 (B-E). RT-PCR analysis confirmed the expression of these retinal progenitor markers and indicates similar expression levels of cells grown in a non-xenogeneic environment when compared to the cells grown using traditional systems (F). Markers of more mature cells are not expressed at this stage, including CRX and BRN3, indicating the temporal specificity of these retinal progenitor cells. After manual isolation of retinal neurospheres based on morphological cues, similar levels of expression were observed between samples for each of the indicated transcription factors (G).
Fig. 2.5. Heparin-independent retinal progenitor cell specification of hiPSCs. hiPSCs grown under non-xenogeneic conditions were further evaluated after 25 days of differentiation to evaluate the necessity of heparin to confer a retinal progenitor cell fate. Isolated retinal progenitor neurospheres previously grown in the presence (+heparin) or absence (-heparin) of heparin demonstrated robust expression of the retinal progenitor marker CHX10 (A). The expression of CHX10 was not significantly altered due to the presence or absence of heparin, with greater than 90% of all aggregates and cells expressing CHX10 (B).

factors such as CHX10 and PAX6 (Figure 2.4 B, C) as well as LHX2 and SIX6 (Figure 2.4 D, E) within the same field of cells, further supporting the retinal progenitor nature of these cells.

The absence of expression of mature retinal genes such as CRX and BRN3, along with expression of transcription factors such as CHX10 underscored the retinal progenitor nature of these populations as expected, with the expression of these transcription factors expressed similarly across all three systems (Figure 2.4 F). No significant differences in gene expression patterns across the three growth conditions were observed,
confirming the similarity of the XF system to traditional MEF and FF systems (Figure 2.4 G). Furthermore, the retinal progenitor marker CHX10 remained highly expressed in the presence or absence of heparin in neural induction medium, suggesting a dispensable role for this traditional media supplement (Figure 2.5).

Non-retinal neurospheres were previously demonstrated to possess a forebrain progenitor fate [21]. In the current study, these neurospheres were similarly comprised of neural progenitors possessing an anterior identity (SOX1/PAX6/OTX2-positive) as well as βIII-TUBULIN-positive neurons (Figure 2.6 A-D). As demonstrated by RT-PCR analysis, these neurospheres expressed a full complement of anterior neural transcription factors (Figure 2.6 E). These results demonstrated the ability to enrich for retinal progenitor cells apart from other neural cell types maintained under xeno-free conditions, as previously established for traditional systems. Additionally, expression patterns of SOX1, PAX6 and OTX2 were largely unaffected by XF, FF and MEF systems of differentiation (Figure 2.6 F).

### 2.3.3 Differentiation of mature retinal cell types

For translational relevance leading to clinical applications, it will be necessary to derive retinal cells under non-xenogeneic conditions, including cells of both the RPE and neural retina. In the current study, hiPSCs were therefore directed to become neural retinal and RPE cells under xeno-free conditions, similarly to those previously documented in the MEF and feeder-free systems [21]. Initially, RPE differentiation was induced through modifications to previously established methods [20, 21], and pigmented, hexagonal RPE-like cells were first apparent approximately one month following the start of differentiation.

The number of pigmented cells increased in abundance over next few weeks (Figure 2.7 A). Immunocytochemistry at 50 days of differentiation revealed the expression of
Fig. 2.6. Non-retinal neurospheres retain an anterior neural identity. Non-retinal neurospheres expressed markers indicative of neural progenitor fate such as SOX1 and PAX6 after 25 days of differentiation (A-B). Further analysis of these cells demonstrated their anterior neural phenotype based on the expression of OTX2 (C). At this stage of differentiation, the first expression of neuronal-specific markers such as βIII-tubulin was observed (D). Arrows in C and D indicated that βIII-tubulin-positive neurons had lost the expression of progenitor-associated transcription factors such as OTX2. RT-PCR analysis demonstrated that these cells expressed a variety of anterior neural transcription factors (E). Quantification of immunocytochemistry experiments performed on non-retinal neurospheres revealed similar percentages of cells within aggregates expressing each indicated transcription factor (F).
Fig. 2.7. RPE derived from hiPS cells under xeno-free and traditional growth conditions. Bright field microscopy demonstrated the characteristic pigmented, hexagonal morphology associated with RPE specification (A). This phenotype was first apparent approximately 1 month following the start of differentiation and increased over the next few weeks. hiPSC-derived RPE grown under all three conditions expressed characteristics such as the transcription factor OTX2 as well as the tight junction protein ZO-1 (B). RT-PCR from xeno-free, feeder-free, and MEF cultures similarly expressed a number of RPE-associated genes (C). Following manual isolation and expansion of RPE derived under each growth condition, comparable percentages of cells co-expressing OTX2 and ZO-1 were observed (D).
RPE-characteristic tight junction proteins such as ZO-1 as well as RPE-associated transcription factors, including OTX2 (Figure 2.7 B). RPE from both xeno-free and traditional cultures expressed a full complement of RPE-associated genes such as MITF, OTX2, RPE65, BEST1, EZRIN, CRALBP, ZO-1 and PEDF, as confirmed by RT-PCR (Figure 2.7 C). In addition, Image J and ANOVA analysis demonstrated that the cell counts of OTX2/ZO-1 positive cells were highly similar across XF, FF and MEF systems of growth (Figure 2.7 D).

Beyond the ability to derive RPE cells, cells were observed with morphologies of primitive photoreceptor-like cells in vitro after 60 days of differentiation (Figure 2.8 A). Cells expressed genes associated with varied neuroretinal phenotypes, with numerous cells expressing the photoreceptor precursor-specific transcription factor CRX as well as RECOVERIN, indicative of a photoreceptor-specific fate (Figure 2.8 B). Additionally other cells expressed BRN3, indicative of retinal ganglion cells (Figure 2.8 C). RT-PCR demonstrated the acquisition of advancing features of retinal cells over 50 days of differentiation in a xeno-free environment (Figure 2.8 D). The percentages of cells expressing ganglion cell (BRN3) and photoreceptor (CRX) phenotypes were found to be highly comparable across all three systems of growth (Figure 2.8 E).

2.4 Discussion

The data presented in the current study is the first demonstration of the derivation of multiple retinal cell types from hiPSCs in the absence of xenogeneic components. We established the generation of retinal cell types, including RPE, photoreceptors, and retinal ganglion cells, from hiPSCs under xeno-free conditions similar to those cells maintained and derived by traditional methods of differentiation. Such an ability is a necessary step as hiPSC research is further developed for translational purposes.
Fig. 2.8. Neuroretinal cell types derived under xeno-free conditions. After 60 days of differentiation, a subset of cells acquired morphologies of primitive photoreceptor-like cells in vitro, including a unipolar appearance with one short process, as indicated by arrows (A). At this time point, a subset of cells expressed genes associated with photoreceptor cells, including CRX and RECOVERIN (B). Other cells expressed markers specific to retinal ganglion cells such as the transcription factor BRN3 (C). RT-PCR analysis indicated the expression of genes specific to differentiated retinal subtypes including CRX, whereas the retinal progenitor marker CHX10 is no longer expressed in these cells (D). Aggregates of cells after 60 days of differentiation exhibited comparable percentages of cells within immunopositive colonies expressing CRX and BRN3, indicative of photoreceptors and retinal ganglion cells, respectively (E).
Several reports have focused upon the ability to derive retinal cell types from hiPSCs, with prospects for future therapeutic approaches for patients with devastating blinding disorders. However, most of these studies have relied upon xenogeneic culture systems utilizing media containing animal products and/or mouse feeder cells to support the growth of hiPSCs [18–21, 23, 49, 53, 54, 107, 138]. The ability to derive retinal cells from hiPSCs under non-xenogeneic conditions has profound implications for future approaches to the treatment of retinal degenerative disorders, including age-related macular degeneration, retinitis pigmentosa and glaucoma. Recent reports have focused on the ability to derive RPE cells in a less xenogeneic culture environment [159]. This is of particular importance as the first clinical trials for human embryonic stem cell-based products are underway for the potential treatment of AMD [14]. Although such studies have successfully generated RPE cells, these systems have remained somewhat xenogeneic through the maintained use of serum and other animal components, raising the risk of zoonosis. Furthermore, studies to date have excluded the ability to derive cells of the neuroretina, including photoreceptors and retinal ganglion cells [159].

To build upon previous studies and establish a truly non-xenogeneic system with which to derive retinal cells from hiPSCs, we sought to maintain a xeno-free environment in our system with focus on both media and substrate components. Firstly, synthetically-coated culture plates, a defined, feeder-free alternative for the growth of hiPSCs, were utilized. Secondly, media devoid of xenogeneic components was used to specify neural and retinal fates. The ability to derive retinal phenotypes under non-xenogeneic conditions also has important implications for the development of stem cell-based approaches to a variety of disorders, as many groups have demonstrated the ability to derive a variety of retinal cell types following similar differentiation paradigms [16, 19].
The results presented in this study offer numerous advantages over previously described approaches to derive retinal cells from hiPSCs. First, the non-xenogeneic system established within this manuscript is completely chemically defined; whereas previous efforts to establish a non-xenogeneic culture system often relied upon the use of serum or knockout serum-containing medium at some stage of the differentiation process [159,160]. The lack of animal sera throughout this protocol helps to create a more reproducible culture system, as significant variability often exists between lots of animal-derived products. Additionally, beyond the use of commercially available media supplements, this method does not require the use of additional exogenous growth factors which may complicate efforts to establish a non-xenogeneic, more reproducible culture system. Furthermore, as previously demonstrated under traditional culture systems [21], we establish the ability to identify and enrich for retinal progenitor cell populations based upon morphological characteristics and here demonstrate this capability under non-xenogeneic conditions. Such an ability to derive highly enriched populations of retinal progenitor cells under non-xenogeneic culture conditions is an essential step for the development of translational applications of hiPSCs.

Previous studies using similar neural and retinal differentiation protocols have often used heparin as a component of the differentiation medium. However, the necessity of heparin in these media has not been thoroughly tested. For the establishment of a non-xenogeneic culture system, proteins of animal origin must be either removed or replaced with suitable alternatives. The experiments described in this study demonstrate that although heparin has been widely used for neural and retinal specification media, the presence of heparin is perhaps superfluous and may likely be eliminated as needed in future studies. Thus, the studies described within this report not only demonstrate the ability to derive retinal cell types under non-xenogeneic culture conditions, but the results also underscore the ability to use such an approach to the development of refinements to existing protocols.
Beyond the implications for advanced therapeutic approaches to retinal disease, the establishment of a non-xenogeneic system with which to maintain and differentiate hiPSCs also has significance for the use of these cells as an in vitro model of human development or for disease modeling. Previous studies have demonstrated the ability of hiPSCs to recapitulate each of the major stages of retinogenesis, yielding each of the major cell types of the retina. However, questions remained about how well such systems faithfully recapitulate in vivo development, as these systems have relied upon factors that would not be found in a human in vivo system, including mouse cells as well as animal serum and other proteins. With the establishment of a non-xenogeneic system with which to direct the differentiation of hiPSCs to a retinal fate, future studies of human retinogenesis utilizing hiPSCs will likely be more easily translatable to an in vivo environment, and may more faithfully recapitulate disease-associated phenotypes associated with patient-specific samples. Furthermore, such an ability to derive retinal cell types under non-xenogeneic conditions will also simplify efforts to utilize hiPSCs for pharmacological screening, as these cells are more likely to faithfully recapitulate the in vivo environment due to the lack of xenogeneic components. Normalizing culture conditions will also make subtle phenotypes more easily attributable to underlying factors that may otherwise be overlooked due to inherent variability associated with xenogeneic conditions. Thus, investigators will be able to more reliably draw comparisons between patient-derived cell lines and models.

These results establish that hiPSCs can be specified to differentiate into mature neural cell types such as retinal neurons under non-xenogeneic conditions. Such results have important implications as new stem cell-based approaches are developed for the treatment of human disease, including those proceeding to clinical trials for the potential treatment of AMD [14]. Before cells grown under non-xenogeneic conditions can be utilized in therapeutic applications, other precautions will be necessary. Existing cell lines need assayed for xenogeneic material that had been previously acquired. It may therefore be advantageous to derive new lines of hiPSCs under non-xenogeneic
conditions, as described previously for hiPSCs [154]. Additionally, routine culturing of these cells in a research lab does not afford the same level of protection for a patient as those cells grown under good manufacturing practices (GMP) [161]. Before the translation of this research to therapeutic applications, it will be advantageous and perhaps necessary to expand the results presented here to include the derivation and differentiation of these cells under GMP-compliant environmental conditions. The results presented here describe for the first time chemically-defined, non-xenogeneic culture conditions for deriving retinal cell types as described previously [20].

2.5 Conclusions

In summary, the results of this study demonstrate the ability to differentiate hiPSCs into a variety of retinal cell types under non-xenogeneic culture conditions. This study represents the first demonstration of non-xenogeneic differentiation of hiPSCs into neural retinal cell types such as photoreceptors and retinal ganglion cells, which is likely to have important implications for the treatment of diseases such as AMD and glaucoma. Of importance, no marked differences in the maintenance and differentiation of hiPSCs into retinal cells were observed between each of the three culture conditions. The results of this study also highlight the applicability of non-xenogeneic growth and differentiation of hiPSCs to other cellular lineages for translational applications. While additional studies are still necessary before widespread application of hiPSCs for translational applications, the current study serves to establish the feasibility of non-xenogeneic growth and differentiation of hiPSCs for applications related to retinal development and disease.
3. ROBUST DIFFERENTIATION OF MRNA-REPROGRAMMED HUMAN INDUCED PLURIPOTENT STEM CELLS TOWARDS A RETINAL LINEAGE

3.1 Introduction

With the ability to differentiate into any cell type of the body, human induced pluripotent stem cells (hiPSCs) have received a great deal of attention in recent years for applications in both basic and translational fields of research [21,79,100,143,162–166]. This is particularly true as it applies to retinal differentiation of hiPSCs, where a number of studies have demonstrated the ability to effectively utilize hiPSCs as a novel in vitro model of human retinogenesis [20–22,26,55,85], including the generation of retinal-like structures from hiPSCs [21,27–29,135]. Furthermore, when derived from patient samples with known genetic determinants of retinal disease, hiPSC-derived retinal cells have proven to be a promising in vitro model for studies of disease progression [21,59,83,106,167,168], while some studies have also demonstrated the potential of hiPSCs as an unlimited source for cell replacement strategies, particularly in models of retinal degeneration [9,15,56,169–171].

Despite the considerable advances that have been demonstrated utilizing hiPSCs, a number of hurdles exist before widespread use of these cells for both basic and translational retinal research. Among these hurdles include the method by which hiPSCs were originally reprogrammed from patient cells. Traditionally, reprogramming transcription factors were retrovirally delivered to patient cells to yield colonies of hiPSCs [3,5,143,172], with many of these lines of hiPSCs currently widely utilized in stem cell research [20–22,26,29,139]. While these approaches established the proof
of principle that hiPSCs could effectively be generated from somatic cells, they also
possessed certain shortcomings that made them less than ideal candidates for trans-
lational applications. Firstly, due to the integrating nature of retroviral DNA into
the host cell genome, concerns exist regarding the integration site within the genome
of the host cell [173–176]. Furthermore, these reprogramming factors were typically
driven by strong constitutive promoters. While many studies have demonstrated the
silencing of these transgenes after the establishment of new lines of hiPSCs [3, 143],
the potential exists for deleterious constitutive expression of these genes. Efforts have
since focused on improving these methods through strategies such as excisable lentivi-
ral vectors or non-integrating episomal vectors [172,177–180], which still rely on DNA
transfection, or direct protein transduction [181,182], which has proven to be difficult
due to insufficiencies obtaining required quantities of protein, and still results in low
reprogramming efficiencies.

More recently, the use of synthetic mRNAs for transfection and reprogramming has
been demonstrated to be an effective and efficient strategy for the generation of
hiPSCs [149,183], but this approach was not utilized with the purpose of retinal
differentiation from these cells. Thus, efforts were undertaken to test the ability
of mRNA-reprogrammed hiPSCs to be effectively differentiated to a retinal lineage.
Lines of hiPSCs were generated via mRNA transfection of pluripotency factors and
in parallel, other lines of hiPSCs were generated through retroviral reprogramming
strategies from the same source material. mRNA reprogrammed hiPSCs (miPS cells)
were tested for their ability to generate retinal cells as has been previously demon-
strated for traditional reprogramming strategies. These results were compared to
retrovirally-derived hiPSCs (riPS cells) to test for the ability of mRNA-reprogrammed
hiPSCs to effectively and efficiently yield retinal progeny in a predictable temporal
and stepwise fashion. The results presented here are the first to explore the ability
of mRNA-reprogrammed hiPSCs to be directed to a retinal phenotype and supports
the use of mRNA-reprogrammed hiPSCs as an effective and important alternative to traditional reprogramming strategies for subsequent retinal differentiation.

3.2 Methods

3.2.1 Reprogramming of fibroblasts to pluripotent state

Human foreskin fibroblast cells (BJ fibroblasts, Stemgent) were maintained and expanded in media consisting of DMEM with 10% fetal bovine serum, MEM non-essential amino acids, and penicillin-streptomycin. For reprogramming, approximately 50,000 fibroblasts were seeded into each well of a 6-well plate, with this day designated as “Day 0”, and cells were allowed to adhere overnight. The next day, cells were switched to Pluriton reprogramming medium (Stemgent) and mRNA reprogramming was initiated via chemical transfection of synthetic mRNAs encoding for reprogramming factors into fibroblasts with Stemfect transfection reagent (Stemgent) beginning on Day 1, with daily transfections and medium changes thereafter through Day 14. To enhance reprogramming efficiency via mRNA transfection, a microRNA booster kit (Stemgent) was similarly chemically transfected on Day 0 and Day 4 as per manufacturers instructions. Colonies of hiPSCs generated via mRNA reprogramming were observed within 3 weeks following the first mRNA transfection, at which point medium was changed to mTeSR1 (StemCell Technologies). For retroviral reprogramming, viral particles encoding for reprogramming factors (Stemgent) were added to fibroblasts the day after plating. Fibroblast medium was replaced the next day and every other day thereafter until Day 6, at which point medium was changed to mTeSR1. Colonies of hiPSCs generated via retroviral reprogramming were visible within the first 3 weeks following viral infection. Typically, 5-10 colonies of newly reprogrammed iPSCs were identifiable in each well via both methods of reprogramming.

To establish individual lines of hiPSCs from both mRNA and retroviral reprogramming strategies, cells were initially live-cell stained with an antibody against Tra-1-60.
(Stemgent) and pluripotent colonies were visualized via the green fluorophore conjugated to this antibody. Upon prospective identification of successfully reprogrammed cells, individual colonies were manually isolated with a pipette and transferred to a matrigel-coated well of a 6-well plate. Isolated colonies were maintained in mTeSR1 medium and passaged as needed to expand and establish as new, individual lines of hiPSCs.

3.2.2 Maintenance of undifferentiated cells

Three mRNA-reprogrammed lines of hiPSCs (designated miPS-2, miPS-4, and miPS-6) and three retrovirally-reprogrammed cell lines (riPS-1, riPS-2 and riPS-4) were maintained in the undifferentiated state as previously described [22, 26, 184]. Briefly, hiPSCs were maintained on six well plates coated with matrigel (BD Biosciences) and supplemented with mTeSR1 medium (StemCell Technologies). Medium was replaced on a daily basis and upon reaching confluence, undifferentiated colonies were passaged using dispase (2 mg/ml, Life Technologies). hiPSCs were typically passaged every 5 days at a ratio of 1:6.

3.2.3 Retinal differentiation of hiPSCs

Each of the six lines of hiPSCs, either mRNA or retrovirally derived, were directed to a retinal fate using previously established protocols [20–22, 26, 28, 55]. Briefly, differentiation was initiated via the formation of embryoid bodies which were transitioned into a neural induction medium (NIM: DMEM/F12, N2 supplement, MEM nonessential amino acids, 2µg/ml heparin) over the first 3 days of differentiation as previously described [22, 26]. Embryoid bodies were plated onto 6-well plates after 6 total days of differentiation with the addition of 10% fetal bovine serum to allow for attachment. The next day, fetal bovine serum was removed and cells were maintained in NIM until Day 16 of differentiation. At this point, cell clusters were lifted as previously described and maintained in suspension in retinal differentiation medium (RDM: DMEM/F12
with B27 supplement and antibiotics). Optic vesicle-like (OV-like) structures were readily identifiable after 20 total days of differentiation and manually isolated based on morphological features as previously described. OV-like structures were allowed to further grow in RDM until a total of 70 days of differentiation, at which point specific retinal cell types could be identified. Alternatively, RPE were derived from hiPSCs as previously described.

### 3.2.4 RPE differentiation

RPE were derived from lines of hiPSCs as previously described [21, 22, 26]. Briefly, after a total of 16 days of differentiation as described above, cells were allowed to remain adherent and medium was changed to RDM. After a total of approximately 2-3 months of differentiation, pigmented patches of RPE were readily observable in the dishes, and suitable areas of RPE cells were micro-dissected with a pipette tip. Clusters of RPE cells were then plated onto poly-ornithine/laminin-coated coverslips. RPE plated on coverslips were expanded in RDM with addition of FGF2 and EGF (both 20 ng/ml) for approximately 1 week, at which point cells were maintained in RDM alone for approximately two more weeks to allow cells to stop proliferating and mature further.

### 3.2.5 Immunocytochemical analysis

Samples were analyzed by immunocytochemistry as previously described [20–22]. Briefly, samples were collected at known stages of retinal differentiation and fixed with 4% paraformaldehyde for 30 minutes, followed by 3 washes with PBS. Primary antibodies (Tables 2.1 and 2.2) were diluted in a solution consisting of 0.1% Triton X-100 and 5% donkey serum in PBS and cells were incubated overnight at 4°C. The next day, secondary antibody was diluted at 1:1000 and added to the cells along with DAPI for one hour at room temperature. Images were later captured on either a
Leica DM5500 fluorescence microscope with a Hamamatsu Orca-R2 digital camera or a confocal/two-photon Olympus Fluoview FV-1000 MPE system.

3.2.6 Reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative RT-PCR

RT-PCR and quantitative RT-PCR (qRT-PCR) was performed as previously described [21, 22, 26]. Briefly, RNA was extracted with the PicoPure RNA Isolation Kit (Applied Biosystems), followed by cDNA synthesis with iScript cDNA synthesis kit (Bio-Rad). PCR amplification was performed using GoTaq qPCR Master Mix (Promega) for 35 cycles and analyzed on 2% agarose gels. For qRT-PCR analysis, cDNA was amplified with pre-designed primers for β-actin (Hs00969077), RAX (hs00429459), CHX10 (Hs01584047), CRX (Hs00230899) and TaqMan Universal Master MiX II (Life Technologies). For OCT4, primers were designed using the NCBI gene sequence and amplified with SYBR green PCR master mix (Life Technologies). Each sample was run in triplicate and a minimum of three samples were used to quantitatively assess the mRNA expression across all cell lines. A complete list of all primer sequences can be found in tables 2.3 and 2.4.

3.2.7 Data quantification

A minimum of three samples from each cell line were plated onto poly-ornithine/laminin-coated coverslips and immunocytochemistry was performed as described above at indicated time points of differentiation. For each time point, representative images from random areas of the coverslip were acquired from an average of at least 2500 cells per cell line for each marker indicated and the number of antibody-stained nuclei was quantified using Image J software. Cell counts are expressed as mean ± S.E. and the percentage of cells expressing indicated markers was compared to the total number of cells as indicated by total DAPI-stained nuclei. To determine statistically signifi-
cant differences between samples, ANOVA analyses were performed using GraphPad Prism software, with significant differences identified at a p-value of less than 0.05.

3.3 Results

3.3.1 Reprogramming of human fibroblasts to pluripotency

The effective reprogramming of somatic fibroblast cells to a pluripotent state has been routinely accomplished through the introduction and expression of a core set of transcription factors [3, 5, 143, 149, 172, 179, 180]. Traditionally, these genes have been delivered through retroviral methods, although newer non-integrating methods, including mRNA-based reprogramming, hold tremendous potential for a variety of basic and translational applications. However, such methods have yet to be described with the subsequent goal of deriving retinal cells. Thus, efforts were undertaken to establish the ability of hiPSCs to effectively yield retinal cell types from somatic fibroblasts reprogrammed to pluripotency by mRNA-reprogramming methods.

Human fibroblast cells were grown in culture and either transfected with synthetic mRNA or, as a control and point of comparison, infected with retroviral particles encoding for pluripotency transcription factors. In addition, these pluripotency cocktails included a nuclear green fluorescent protein (nGFP) reporter for mRNA reprogramming or a green fluorescent protein (GFP) reporter for retroviral reprogramming (Figure 3.1 A-B) to identify properly transfected/infected cells. Within the first 3 days following transfection/infection, nGFP expression was observed in nearly all fibroblasts (95.46 ± 2.81%) transfected with mRNA, while a fraction of fibroblasts in parallel cultures exhibited GFP (28.67 ± 4.14%) after infection with retrovirus (Figure 3.1 C-D), when compared to untransfected cells as a negative control. Within the first three weeks after transfection/infection, profound morphological changes were apparent in a subset of fibroblasts, in which the elongated, spindle-like morphology typical of fibroblasts was lost in favor of a more compact, rounded appearance typical
Fig. 3.1. Reprogramming of fibroblast samples to pluripotency. Human fibroblasts were transfected with daily doses of mRNA encoding for pluripotency reprogramming factors, as well as a nuclear GFP reporter (A). Similarly, other cultures of fibroblasts were infected with retroviruses of the above transcription factors, including GFP reporters (B). Efficiency of gene delivery was approximately $95.46 \pm 2.81\%$ for mRNA methods, and $28.67 \pm 4.14\%$ for retroviral methods (C-D). Within 3 weeks following the delivery of these reprogramming factors, compact colonies indicative of putative hiPSCs were observed (E-F), which were identified by the expression of the pluripotency-associated cell surface marker Tra-1-60 (G-H). These colonies were further isolated to generate stable lines of hiPSCs.
of pluripotent cells (Figure 3.1 E-F). To further identify these presumptive hiPSCs as pluripotent, live cell staining confirmed the expression of the cell surface marker Tra-1-60 specifically on these compact colonies of cells (Figure 3.1 G-H).

To establish discrete, individual lines of hiPSCs, colonies of Tra-1-60-expressing cells were manually isolated and expanded in culture to further characterize their pluripotent nature. Three new lines of hiPSCs were generated from each reprogramming method, designated as either miPS (mRNA-reprogrammed induced pluripotent stem) cells or their control counterparts, riPS (retrovirus-reprogrammed induced pluripotent stem) cells. Numerous characteristics have traditionally been utilized to confirm pluripotency, including the expression of both transcription factors as well as cell surface markers. Thus, efforts were undertaken to demonstrate a full complement of these pluripotency factors in each of these newly established lines of hiPSCs. Each cell line exhibited robust expression of pluripotency-associated transcription factors such as OCT4, SOX2, and NANOG, as well as cell surface markers SSEA-4, Tra-1-60, and Tra-1-81 (Figure 3.2). In the pluripotent state, little variability was observed between each of the newly generated lines of hiPSCs (Figure 3.2), with miPS cells expressing pluripotency-related characteristics at levels equivalent to riPS cells.

Beyond the expression of factors characteristic of pluripotency, true pluripotent cells possess the ability to give rise to all cell types of the body. Traditionally, this has been demonstrated through the spontaneous differentiation of these cells followed by identification of differentiated progeny from each of the three germ layers [3,5,21,143, 185]. To demonstrate the pluripotent nature of these newly generated hiPSCs, each line was allowed to spontaneously differentiate as embryoid bodies. After three weeks of growth and differentiation under these conditions, cell types of each of the three germ layers were observed, as evidenced by the expression of β-III tubulin, smooth muscle actin, and α-fetoprotein representing the ectoderm, mesoderm, and endoderm, respectively (Figure 3.3).
Fig. 3.2. Establishment of lines of hiPSCs through mRNA and retroviral reprogramming methods. mRNA-reprogrammed lines of hiPSCs (miPS-2, miPS-4 and miPS-6) and retrovirally reprogrammed cell lines (riPS-1, riPS-2 and riPS-4) expressed a full complement of pluripotency-associated characteristics. Quantification of immunocytochemistry results indicated that while some lines of hiPSCs varied in expression levels of SOX2 and NANOG, mRNA-reprogrammed hiPSCs overall did not overtly differ from their retroviral counterparts.*

* = p < 0.05
Fig. 3.3. miPS and riPS lines generate cells of all three germ layers. To confirm the pluripotent nature of these newly generated miPS and riPS lines, differentiation was performed via the formation of embryoid bodies to further assess the pluripotent nature of these cells. All lines of hiPSCs were analyzed for germ layer markers after 3-4 weeks of differentiation in medium consisting of DMEM/F12 and 20% knockout serum replacement. Ectodermal cells were identified by the expression of βIII-tubulin, mesodermal cells were identified by the expression of smooth muscle actin, and endodermal derivatives were identified by the expression of α-fetoprotein.
3.3.2 Neuroretinal differentiation from hiPSCs

The derivation of retinal cell types from a pluripotent cell source such as hiPSCs necessitates a stepwise progression through various stages of development, including the acquisition of a primitive anterior neuroepithelial/eye field phenotype followed by the optic vesicle and optic cup stages of development, before eventually yielding the major cell types of the retina. Previous studies have demonstrated that not only are each of these stages of retinogenesis achievable during the differentiation of hiPSCs, but this differentiation also occurs in a manner in which each of these major stages of retinogenesis occur in a predictable and readily identifiable fashion [20,21,28,29,186]. As such, the ability of mRNA-derived hiPSCs to differentiate in such a manner that closely recapitulates these major stages of retinogenesis was explored.

All lines of hiPSCs derived through mRNA and retroviral reprogramming methods were directed to differentiate to a primitive anterior neuroepithelial/eye field fate as previously described. Within 10 days of differentiation, robust expression of markers associated with this developmental state were observed in all cell lines, including PAX6, SOX1, OTX2, and LHX2 (Figure 3.4). Quantification of the percentage of cells expressing these transcription factors indicated that mRNA-reprogrammed hiPSCs were equally capable of differentiating toward this anterior neuroepithelial stage as compared to their retrovirally-derived counterparts, and did so in a highly efficient manner.

After the acquisition of a primitive anterior neuroepithelial/eye field phenotype, the next major stage in retinal development is the subsequent acquisition of an optic vesicle/optic cup-like fate. Previous studies with human embryonic stem cells and virally-derived hiPSCs have indicated that these cells readily give rise to retinal progenitors analogous to the optic vesicle/optic cup stages of retinal development within the first 30 total days of differentiation [21,22,26,28,29,55]. Furthermore, these retinal
Fig. 3.4. Establishment of a primitive neural fate from mRNA and retrovirally-reprogrammed cell lines. miPS and riPS cell lines expressed primitive neuroepithelial markers including PAX6 and SOX1 at high efficiency within the first ten days of differentiation. Furthermore, all hiPSC lines also expressed regional markers including OTX2 and LHX2, indicative of acquisition of anterior neural identities. Immunocytochemistry analysis demonstrated significant differences in expression levels of PAX6, SOX1 and LHX2 among some miPS and riPS lines, however no correlation was observed between mRNA versus retroviral method of reprogramming. * = p < 0.05
Fig. 3.5. Differentiation of miPS and riPS cells to a retinal lineage. Following the establishment of an anterior neural fate, a subset of cells acquired a definitive retinal progenitor fate within 30 days of differentiation, as confirmed by the expression of retinal progenitor markers including CHX10 and PAX6. The highly proliferative nature of these cells was demonstrated by the expression of Ki-67. Importantly, cells lost the expression of SOX1, a marker found in many anterior neural cell types but lost within retinal cells. Immunocytochemistry analysis demonstrated some variability in CHX10 expression among hiPSC lines irrespective of reprogramming method while expression of PAX6 was consistent across all cell lines. * = p < 0.05
progenitor cells were found to be organized into individual neurospheres, allowing for the isolation and enrichment of hiPSC-derived retinal progenitor cells. Correspondingly, miPS cells were directed to differentiate toward an optic vesicle-like stage and manually isolated and enriched as previously described. Upon analysis of these retinal progenitor cells, robust expression of retinal progenitor-associated markers such as CHX10 and PAX6 were observed (Figure 3.5). Interestingly, unlike after 10 days of differentiation, the expression of SOX1 was nearly completely absent in all cell lines after 30 days of differentiation, indicating the commitment of these cells to a retinal lineage. Quantification of these results indicated that miPS cells were capable of acquiring this optic cup-like phenotype in a highly efficient manner, with nearly 90% of all cells expressing CHX10 in some cell lines following isolation of retinal progenitor neurospheres. Importantly, these miPS cells were capable of yielding CHX10-positive and PAX6-positive retinal progenitor cells as well as or greater frequency than their retroviral counterparts.

3.3.3 Differentiation of specific retinal cell types

Retinal progenitor cells of the optic vesicle and optic cup have the ability to give rise to all of the cell types of the retina, and do so in a precise and temporal fashion [37, 47, 187]. Similarly, hiPSC-derived retinal progenitor cells have previously been demonstrated to give rise to all of the major cell types of the retina in an orderly manner as well. Thus, efforts were undertaken to determine and quantify the ability of both mRNA and retrovirus-derived hiPSCs to yield cell types of the retina. Initially, within the first 30 days of differentiation, pigmentation associated with the onset of retinal pigment epithelium (RPE) differentiation could be observed as previously described [7, 56, 57, 60, 80, 81, 84, 126, 139, 188–190]. These RPE cells became more abundant over the next 1-2 months and could eventually give rise to expandable monolayers of pigmented, hexagonal RPE-like cells from all cell lines tested (Figure 3.6).
Fig. 3.6. Derivation of retinal pigmented epithelium from mRNA and retroviral reprogrammed cell lines. Retinal Pigmented Epithelium (RPE) was derived from lines of miPS and riPS within 60-90 days of differentiation. Brightfield images demonstrating pigmented patches of hexagonal cells characteristic of RPE were observed in all lines of hiPSCs that were capable of proliferation and expansion.

Alternatively, CHX10-positive OV-like structures give rise to cells of the neural retina, particularly the more early-born cell types such as retinal ganglion cells and developing photoreceptors (Figure 3.7), as has been previously documented [21,22,26,28,29]. Interestingly, all lines of miPS cells were able to generate CRX-positive photoreceptor-like cells at statistically similar efficiencies, correlated with the ability of retroviral-derived cells to yield photoreceptor-like phenotypes.
Fig. 3.7. Generation of retinal cell types from hiPSCs. After approximately 70 days of differentiation, cells were analyzed for the expression of markers of retinal ganglion cells (BRN3/MAP2) or retinal photoreceptors (CRX/Recoverin). All cell lines were capable of generating these retinal cell types, although significant variability was observed in the expression of BRN3 among some miPS and riPS cell lines ( * = p < 0.05, ** = p < 0.01, *** = p < 0.005 ).
Additionally, all miPS and riPS cells lines expressed of genes associated with retinal ganglion cells, photoreceptors and interneurons of the retina, as demonstrated by RT-PCR experiments (Figure 3.8). However, variation in their ability to produce BRN3-positive RGCs was observed in both miPS and riPS cell lines, with some lines capable of yielding nearly half (49.13 ± 2.95%) BRN3-positive retinal ganglion cells following enrichment for OV-like structures.

3.3.4 miPS and riPS cells differentiate to a retinal lineage in a developmentally regulated manner

The ability to derive retinal cells from hiPSCs allows for the developmental investigation into the earliest stages of retinogenesis. In order for lines of hiPSCs to serve as a model for retinal development, however, it is essential that these cells are directed to differentiate in a manner which closely mirrors known stages of retinogenesis. Thus, efforts were undertaken to demonstrate the stepwise acquisition of retinal characteristics from both mRNA and retroviral-derived hiPSCs through known stages of retinal development (Figure 3.9). Samples were collected at different stages of differentiation and gene expression profiles across all cell lines were evaluated via qRT-PCR experiments.

Similar trends of differentiation were observed across cell lines, with the expression of the pluripotency marker OCT4 gradually lost over the first ten days of differentiation. This loss of OCT4 expression coincided with an increased expression of retina-specific transcription factor RAX, thereby indicating the establishment of the eye field. After 30 days of differentiation, the sustained expression of RAX led to the establishment of the OV-like structures, as indicated by the onset of the retinal progenitor marker CHX10. These retinal progenitor cells later gave rise to various retinal phenotypes, including the expression of CRX, indicative of developing photoreceptors, by 70 days of differentiation.
Fig. 3.8. Derivation of other retinal cell types from hiPSCs. The ability of miPS and riPS cell lines to express other retina-associated markers was evaluated by RT-PCR. All cell lines expressed the full complement of genes typically associated with retinal ganglion cells (BRN3, ISLET1, RBPMS, γSYNUCLEIN) and photoreceptors (CRX, NEUROD4, NRL) (A and B respectively). Furthermore, miPS and riPS cell lines also generated bipolar, horizontal and amacrine neurons of the retina, as demonstrated by the expression of VSX1, PROX1 and PARVALBUMIN respectively (C).
3.4 Discussion

The ability to direct the differentiation of hiPSCs to a retinal lineage has been the subject of intense interest and numerous studies in recent years, and has generated considerable enthusiasm for the study of human retinal development and disease progression [19–22,26,29,55,59,83,106,167,168,191]. However, such efforts are often complicated by numerous factors related to the culture of hiPSCs, including the method by which somatic cells have been reprogrammed to a pluripotent state. The results presented within this study for the first time demonstrate the feasibility of deriving retinal cells from mRNA-reprogrammed hiPSCs and will facilitate future efforts toward the development of translational and therapeutic applications of hiPSC-derived retinal cells.

Traditionally, hiPSCs were reprogrammed from a fibroblast source through the use of viral vectors to deliver DNA encoding for pluripotency-associated transcription factors [3,5,185]. While these approaches were straightforward and effective, the use of viral vectors was also associated with numerous undesirable features, particularly for the future translational application of hiPSCs and their differentiated progeny. First, the use of DNA vectors has often been accomplished through the use of constitutive promoters driving the expression of pluripotency transgenes. While such constitutive expression may assist in the efficient reprogramming of fibroblasts to an hiPSC fate, it may also hinder efforts to direct the differentiation of resultant hiPSCs as well. Furthermore, viral delivery of reprogramming vectors is also associated with the risk of insertional mutagenesis, as these vectors incorporate into the host genome in a random fashion [173–176]. The effects of these genomic insertions may interrupt certain genes necessary for differentiation to desired cellular lineages, or even disrupt tumor suppressor genes leading to uncontrolled growth of cells.
Fig. 3.9. In vitro retinal differentiation recapitulates known stages of retinogenesis. All lines of hiPSCs were assessed for expression of stage-specific genes during the course of retinal differentiation (A). Quantitative RT-PCR analyses demonstrated that while the expression of pluripotency factor OCT4 decreased upon differentiation, transcription factors RAX and CHX10 were expressed at slightly later timepoints and were largely retained up to 70 days of differentiation (B-D). As expected from in vivo studies of retinogenesis, expression of photoreceptor-specific marker CRX was only seen at 70 days of differentiation (E). Overall, results demonstrated that all miPS and riPS cell lines recapitulated stage-specific patterns of gene expression typically associated with human retinogenesis.
More recent efforts to generate hiPSCs have focused upon methods by which to minimize the likelihood of the above issues, while still maintaining or improving reprogramming efficiency. Perhaps most notably, the use of non-viral methods such as through the transfection of episomal vectors has become more widely utilized in recent years [172, 192]. Episomal vectors are ostensibly non-integrating in nature and thus, should eventually be lost by cells after repeated passaging. Such an approach would minimize the risk of insertional mutagenesis as these vectors would not integrate into the host genome. Furthermore, with the eventual loss of these vectors over time, the risk of constitutive expression of pluripotency factors is decreased. However, due to the DNA-based nature of these vectors, one cannot completely eliminate any risk of genomic integration and/or constitutive expression. Thus, a need exists to derive lines of hiPSCs in an efficient manner that is free of the above concerns. The recent advent of mRNA-based reprogramming strategies suitably eliminates these concerns as the DNA transgene-free source would prevent genomic integration, and the mRNA nature of these vectors would be rapidly degraded within the cell.

While the importance and utility of mRNA reprogramming for the generation of hiPSCs has been well-documented [149, 183], the ability of these cells to faithfully recapitulate existing differentiation protocols and yield all retinal cells had yet to be demonstrated. The ability to derive cells of the retinal lineage has important implications for studies of human retinogenesis and the progression of retinal degenerative disorders in patient-derived cells. Furthermore, hiPSC-derived retinal cells have been proposed as an optimal candidate for cell replacement within the nervous system, owing to the ease of accessibility of the retina as well as its highly organized nature [8, 9, 15, 169, 193]. In fact, recent efforts are working toward clinical applications of human pluripotent stem cells, including hiPSCs, for the potential treatment of retinal degenerative disorders [14, 57, 126]. The efficient derivation of retinal cells from mRNA-reprogrammed hiPSCs represents a significant advance in the development of hiPSCs as effective models of human retinal development and/or disease progression.
However, for many applications it is also important to demonstrate that differentiation protocols to desired cellular lineages are comparably efficient when utilizing mRNA-reprogrammed hiPSCs, if not enhanced, when compared to their viral-derived counterparts.

The results of the current study help to demonstrate that the non-integrating, DNA-free nature of mRNA reprogramming is likely to be an important development for the generation and application of hiPSC-derived retinal cells. Importantly, hiPSCs derived through mRNA reprogramming strategies were demonstrated to yield retinal cells at a similar efficiency compared to their viral-derived counterparts, as significant differences between cell lines could not be readily attributed to the reprogramming strategy. In order to establish the utility and efficiency of retinal differentiation from hiPSCs, it was important to establish proper control lines of hiPSCs as genomic variations between different samples would likely introduce increased variability between lines [21, 194, 195]. Thus, the current study explored the use of hiPSCs derived from both mRNA and retroviral reprogramming strategies by utilizing the identical source material of BJ fibroblasts, which have previously been utilized by several other groups for the derivation of hiPSCs [3, 5, 149, 196]. Thus, any potential differences between cell lines could not be attributed to the origin of cells prior to reprogramming.

While a small degree of variability was observed between some cell lines at each stage of differentiation, no correlation was evident due to reprogramming method. With the intent of determining the ability of mRNA-reprogrammed hiPSCs to give rise to retinal cells compared to their retroviral-derived counterparts, it is important to note that variability between different lines of hiPSCs is well-documented [21, 194, 195], even when some of these cell lines are derived from the same source. Such a degree of variability may be due to subtle differences in the reprogramming process itself, epigenetic factors intrinsic to the heterogeneity of cells within a starting population, or even the degree of transgene expression and genomic integration. Thus, while the
use of BJ fibroblasts for all experiments should reduce variability between cell lines, a
certain degree of variability may be expected apart from the reprogramming method
itself. To account for this type of variability, three different lines of hiPSCs were gen-
erated through each reprogramming method, and all six lines of hiPSCs were differ-
entiated through each stage of retinal specification. Upon reprogramming to hiPSCs
through both reprogramming methods, resultant cells expressed a full complement
of pluripotency-related features and no significant differences in OCT4 expression
were observed between cell lines regardless of reprogramming method, with mini-
mal variability observed in the expression of other pluripotency-associated markers.
Furthermore, upon initial differentiation to a primitive anterior neuroepithelial stage
after 10 days of differentiation, all cell lines were observed to differentiate similarly,
with a high efficiency of neural differentiation across all cell lines.

After a total of 30 days of differentiation, it has been previously demonstrated that
hiPSC-derived retinal progenitor neurospheres may be readily identified and man-
ually enriched, with these cells capable of giving rise to all major cell types of the
retina [20–22,26,28,29,55,85]. Similarly, hiPSCs derived through mRNA reprogram-
ming methods were capable of generating these retinal progenitor neurospheres within
30 days of differentiation, characterized by the widespread expression of CHX10 and
PAX6. Interestingly, whereas a primitive anterior neuroepithelial fate achieved within
the first 10 days of differentiation is associated with high levels of SOX1 expression,
this seems to be largely lost in retinal progenitor cells derived by 30 days of differenti-
ation, indicative of the acquisition of a definitive retinal fate [17,197,198]. Across all
six lines of hiPSCs, no significant differences were observed in the expression of PAX6,
with all cell lines exhibiting high levels of this protein in nearly all cells. The retinal
progenitor-associated transcription factor CHX10 was also found to be expressed in a
majority of cells in all lines of hiPSCs at this stage, although statistically significant
differences did exist between some lines. No correlation was observed in the expres-
sion of CHX10 between mRNA and retroviral-derived lines of hiPSCs, but one line of
mRNA-derived hiPSCs (miPS-4) and one line of retroviral-derived hiPSCs (riPS-4) expressed CHX10 in significantly more cells (nearly 90%) than other cell lines tested. Interestingly, this level of CHX10 expression is consistent with previous studies with the H9 (WA09) human embryonic stem cell line [21, 28]. Those lines of hiPSCs with lower percentages of cells expressing CHX10 are not likely to be less retinal in nature, as further differentiation of these cells yields retinal neurons at similar efficiencies by a total of 70 days of differentiation. Rather, these variations may represent slight differences in the developmental timing of these cell lines during differentiation, with some cells perhaps not yet reaching a CHX10-positive stage or alternatively, some lines of hiPSCs may begin to differentiate past a CHX10-positive retinal progenitor stage and begin to give rise to committed retinal neurons such as retinal ganglion cells, as has been previously described [28].

Within a total of 70 days of differentiation, hiPSCs from both mRNA and retroviral-derived reprogramming methods were capable of giving rise to more committed retinal neurons, particularly retinal ganglion cells (RGCs) and cone photoreceptor-like cells [37, 47, 187]. Interestingly, these are two of the earliest generated retinal cell types in vivo, suggesting that this differentiation paradigm is biased towards the generation of early-born cell types. While no differences were observed in the differentiation of photoreceptor-like cells across all cell lines tested, statistically significant differences did exist in the generation of retinal ganglion cells, with some lines of hiPSCs generating nearly half BRN3-positive RGCs and others as little as 20%. These differences could not be attributed to the reprogramming method itself and are likely due to some intrinsic difference in the ability of each line to give rise to RGCs, although it is important to note that BRN3 is expressed in many but not all RGCs [199]. Thus, these numbers could be an underestimate of RGC differentiation and perhaps some lines give rise to an undetectable number of BRN3-negative RGCs. Regardless, these results underscore the importance of characterizing new lines of hiPSCs for their abil-
ity to give rise to a desired cell type and selecting lines of hiPSCs carefully as some lines may be more appropriate for certain applications than others.

3.5 Conclusion

The overall results of this study demonstrate that mRNA reprogramming to generate hiPSCs will likely prove to be an important approach for the generation of hiPSCs for a variety of translational and potentially clinical applications in the future. The lack of DNA vectors, whether integrating or non-integrating, eliminates the risk of insertional mutagenesis and constitutive expression of pluripotency transcription factors. Thus, mRNA reprogrammed hiPSCs likely represent cells that will be closer to an embryonic stem cell state of pluripotency, and possess numerous features that would distinguish these cells as likely safer options as hiPSCs are brought closer to therapeutic applications. For the retina, this is particularly important as the differentiated retinal progeny derived from both human embryonic and induced pluripotent stem cells are reaching closer to clinical applications for disease modeling [21, 59, 83, 100, 106, 167], pharmacological screening [21, 111, 167], as well as cell replacement [8, 15, 56, 200, 201]. In the near future, it will also be necessary to demonstrate additional levels of safety before the widespread application of hiPSCs for translational purposes. Recent efforts have focused on the elimination of xenogeneic components from media used to maintain and differentiate hiPSCs [26, 90, 159, 190], and these approaches will need to be combined with appropriate reprogramming strategies such as mRNA reprogramming. Furthermore, the cells of origin for reprogramming to a pluripotent fate should be carefully chosen from specific and appropriate patient sources, including for the purposes of disease modeling and pharmacological screening, as well as for eventual cell replacement.
4. ELUCIDATING HUMAN RETINOGENESIS AND DISEASE MECHANISMS WITH STEM CELL DERIVED RETINAL ORGANOIDs

4.1 Introduction

The ability to derive human pluripotent stem cells (hPSCs) from patient-specific sources allows for the generation of unlimited quantities of cells affected by disease processes, which can then be used to model disease progression and develop therapies for degenerative disorders [77, 169, 202, 203]. To date, several studies have focused upon the ability to direct the differentiation of patient-derived hPSCs to retinal cell types for disease modeling, although these efforts have mostly utilized stochastic methods of hPSC differentiation, with retinal cells heterogeneously arranged [11, 59, 69, 87, 90, 106, 108, 204, 205]. As such, these retinal cells lack the organization and maturation typical of the retina, limiting their ability to serve as a model of retinal development as well as retinal disease pathology.

To address this lack of organization, recent efforts have focused on the differentiation of these cells as three-dimensional retinal organoids, where differentiation progresses in a stepwise manner analogous to early stages of retinal development [21, 27–29]. The ensuing retinal cells self-assemble into a tissue-like structure that recapitulates the architecture and patterning of the retina. As such, differentiation within these organoids mimics the developmental timing and organization of the retina, facilitating their use as models of retinogenesis and disease progression.

While some progress has been made to utilize retinal organoids for studying diseases of the outer retina, including photoreceptors and retinal pigment epithelium
[21, 55, 78, 85, 95, 206], the differentiation and characterization of inner retinal neurons such as retinal ganglion cells (RGCs) has been largely overlooked, with no studies to date applying retinal organoids for studies of optic neuropathies. As the primary connection between the eye and the brain, RGCs serve a critical function in visual transduction pathways. Furthermore, their degeneration and loss is characteristic of a number of disorders including glaucoma, with a current incidence of greater than 60 million individuals worldwide [91, 92]. As such, the study of optic neuropathies with hPSCs would be greatly facilitated by the development of retinal organoids that effectively mimic the development and degeneration of the retinal ganglion cell layer.

To address this shortcoming, current efforts were directed to generate retinal organoids in a manner that closely mirrors the development of native RGCs [20–22, 25, 26, 29]. Subsequently, the ability of these retinal organoids to serve as a model of glaucomatous neurodegeneration was investigated through the use of hPSCs derived from a glaucoma patient with an E50K mutation in the Optineurin gene [22]. Interestingly, patient-derived RGCs demonstrated an activation of apoptotic markers with RGC layers, indicative of a glaucomatous phenotype. The results of this study represent the first description of the use of retinal organoids for modeling the development of RGCs, as well as their ability to enhance our mechanistic understanding of pathways leading to glaucomatous neurodegeneration.

4.2 Methods

4.2.1 Maintenance of undifferentiated cells

hPSCs were maintained in an undifferentiated state as previously described [20, 22, 25, 26]. Briefly, three control lines of hPSCs (H9, miPS2 and Six6-GFP) and one E50K patient-derived stem cell line were maintained in 6-well plates coated with Matrigel (BD Biosciences) and supplemented with mTeSR1 medium (StemCell Technologies). Upon reaching approximately 80% confluency, differentiated areas of the plate were
mechanically removed and the remaining colonies were enzymatically passaged with dispase (2mg/ml, Life Technologies) every 5-6 days and replated at a density of 1:6.

4.2.2 Differentiation of hPSCs into retinal organoids

hPSCs were differentiated into a retinal lineage with slight modifications to previously established protocols [20, 26, 29]. To initiate differentiation, colonies of hPSCs were enzymatically lifted to form embryoid bodies (EBs), and slowly transitioned into neural induction medium (NIM: DMEM/F12, N2 supplement, MEM-Nonessential amino acids, 2µg/ml heparin) over the next three days of differentiation, as described previously. EBs were plated at 6-7 days of differentiation on 6 well plates and supplemented with 10% FBS to allow for attachment. Colonies of differentiating cells remained plated for the next 3 weeks and were mechanically dislodged at day 20-24 of differentiation, at which point they adopted retinal organoid morphologies. These organoids could be readily isolated and separated from their forebrain counterparts based on their brighter appearance at 40 days of differentiation and were maintained in 5ml petri dishes up to 70 days of differentiation in retinal differentiation medium (RDM: DMEM, DMEM-F12, B27, MEM-nonessential amino acids, antibiotics). Prolonged growth in suspension included the supplementation of medium with 10% FBS and 2mM Glutamax every other day.

4.2.3 Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

RNA was isolated at indicated timepoints using the PicoPure RNA Isolation Kit (Life Technologies), and cDNA synthesis was performed using the iScript cDNA synthesis kit (BioRad). qRT-PCR was performed using SYBRGreen (Life Technologies) and samples were run in triplicates on an ABI 7300 machine. A complete list of primers can be found in Table 4.1 and all samples were standardized to β-Actin.
Table 4.1.
Primer sequences used for qRT-PCR

<table>
<thead>
<tr>
<th>Gene Amplified</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-ACTIN</td>
<td>GCG AGA AGA TGA CCC AGA TC</td>
<td>CCA GTG GTA CGG CCA GAG G</td>
<td>103</td>
</tr>
<tr>
<td>HuC</td>
<td>GCT GAG CCC ATC ACA GTC AA</td>
<td>GCT GGG TCT GAT GGT GTA GG</td>
<td>124</td>
</tr>
<tr>
<td>ISLET1</td>
<td>GGG ATC AAA TGC GCC AAG TG</td>
<td>GTG ATA CAC CTT GGA GCG GG</td>
<td>78</td>
</tr>
<tr>
<td>MATH5</td>
<td>ACT GCC TTC GAC CGC TTA C</td>
<td>CTG CAG GGT CTC GTA CTT GG</td>
<td>78</td>
</tr>
<tr>
<td>OPN4</td>
<td>ACA CGT CCT GAC ACC CTA CA</td>
<td>GCA ATG GCC ACC CTG TAC TT</td>
<td>88</td>
</tr>
<tr>
<td>PAX6</td>
<td>AGT GAA TCA GCT CGG TGG TGT CTT</td>
<td>TGC AGA ATT CGG GAA ATG TCG CAC</td>
<td>96</td>
</tr>
<tr>
<td>RBPMS</td>
<td>GCC TGC ACT TTA CCC CAG TA</td>
<td>AAG CGG GAT AGG TGA AAG CA</td>
<td>104</td>
</tr>
<tr>
<td>SIX6</td>
<td>GAA GAG AGC GGC GAT GTG G</td>
<td>TTA CTA CCA CCG ATT GCC CT</td>
<td>150</td>
</tr>
<tr>
<td>SNCG</td>
<td>TGG GTG CGG TGG AAA AGA C</td>
<td>TTC TCG GCC ACT GAG GTC AC</td>
<td>127</td>
</tr>
</tbody>
</table>
4.2.4 Immunocytochemistry analysis and data quantification

Samples were analyzed by immunocytochemistry as previously described [21, 22, 25, 26]. Cells were plated on poly-ornithine/laminin coated coverslips and fixed at the indicated timepoints with 4% paraformaldehyde for 30 minutes, followed by three washes with PBS. Alternatively, intact organoids were processed for cryostat sectioning by initial fixation in 4% paraformaldehyde, followed by subsequent washes in 1X PBS. Organoids were then equilibrated overnight in 20% sucrose solution, followed by 30% sucrose solution the next day and subsequently embedded in 30% sucrose + OCT and frozen on dry ice and stored at -80°C until sectioned. Cryostat sections were performed at 12 microns and stored at -80°C until further analysis by immunocytochemistry.

For immunostaining, samples on coverslips or cryostat sections were washed three times with PBS and permeabilized with 0.2% Triton X-100 solution for 10 minutes at room temperature. Samples were subsequently blocked with 10% donkey serum for an hour and primary antibodies were added at the appropriate dilutions in 5% donkey serum and 0.1% Triton X-100 and incubated at 4°C overnight. On the next day, samples were washed three times with PBS to remove the primary antibody and blocked with 10% donkey serum for an hour. Secondary antibodies and DAPI were diluted at 1:1000 and 1:2000 dilution respectively in 5% donkey serum and 0.1% Triton X-100 solution and samples were incubated at room temperature for an hour. Following three washes with PBS, coverslips were mounted on slides with mounting medium while cryostat sections were sealed with a coverslip. Cells were imaged with either a Leica DM500 fluorescence microscope or an Olympus Fluoview confocal microscope.

Cell counts of immunostained control and patient organoids were quantified in a blind fashion using Image-J software. BRN3-positive RGCs were found in the basal layers of organoids and were manually traced in Adobe Photoshop. These were referred
to as the inner organoid area, while the peripheral areas of organoids were referred to as the outer organoid area. Expression of activated caspase-3 was quantified in inner and outer retinal organoid areas with Image J and ANOVA statistical analyses or students t-test were performed with PRISM software, with statistically significant differences identified at a p value of less than 0.05.

4.3 Results

4.3.1 Organization of hPSCs into eye field stage of development

Human pluripotent stem cells (hPSCs) allow for the unprecedented ability to recapitulate the earliest stages of human development, including the specification of the retina from a more primitive neural progenitor source. While recent studies have described the organization of hPSCs into a stratified-retinal like tissue [21, 27, 29, 35], the events that lead to the generation of such retinal organoids from a pluripotent population remain to be explored. Therefore, hPSCs were directed to generate retinal organoids in a stepwise manner, to elucidate the steps involved in retinal fate establishment from a pluripotent source.

For this purpose, hPSCs were first directed to a primitive neural phenotype and subsequently to retinal organoids in a stepwise fashion [20, 22, 25, 26]. As early as 1 week of differentiation, differentiating hPSCs self-organized into distinct anterior neural population within the primitive neuroepithelium. Such anterior neural regions exhibited overlapping expression of SIX6, PAX6, RAX and SOX2, reminiscent of the eye-field stage of development and (Figure 4.1 A-D). Upon further differentiation, cells gradually acquired features of the optic vesicle, as evidenced by the onset of expression of CHX10 within organized populations of neural cells by 2 weeks of differentiation. (Figure 4.1 E-H). While CHX10 expression was initially seen in smaller patches within differentiating hPSCs, it was later organized into distinct areas resembling a sphere/colony, representative of the transition from an eye field stage to the
Fig. 4.1. Organization of hPSCs into eye-field and their subsequent transition into optic vesicle stages of development. Within the first 10 days of differentiation, hPSC-derived cells began to adopt a neural phenotype and expressed SIX6, PAX6, RAX and SOX2, indicative of the eye-field stage of development (A-D). Optic-vesicle marker CHX10 was seen to gradually emerge within organized patches of the eye-field like populations (E-H) and gradually organized into a more three-dimensional shape, indicative of the optic vesicle stage of development (I-L).
optic vesicle stage of development (Figure 4.1 I-L). Overall, the progression of CHX10 expression within differentiating populations of hPSCs provides the opportunity to examine stages of retinogenesis analogous to the emergence of the developing optic vesicle from the anterior neuroepithelium.

4.3.2 hPSC-derived retinal cells organize apart from forebrain populations

As vertebrate retinogenesis occurs during primitive stages of the development of the nervous system, previous studies have demonstrated that the optic vesicle is specified in parallel with other neural structures including the forebrain from the developing diencephalon [37,156]. However, this process occurs very early in human gestation, and steps involved specification of a retinal fate apart from these other neural populations remains elusive. However, the use of hPSCs allows for insight into these early cell fate determination events. Upon the specification of anterior neuroepithelium from a pluripotent source, differentiating colonies of hPSCs organized into two distinct neural populations after 25 days of differentiation, with some exhibiting retinal features while others were reminiscent of the developing forebrain. Retinal cells exhibited a more three-dimensional appearance, and acquired a defined boundary within their colonies, which was absent in nascent forebrain colonies (Figure 4.2 A, D).

To better understand retinal fate specification, a retinal lineage GFP reporter (SIX6-GFP) was used to distinguish primitive retinal populations from forebrain populations. Within 3 weeks of differentiation, optic vesicle-like colonies of hPSCs were demonstrated to express retinal markers CHX10 and LHX2 that co-localized with the GFP expression (Figure 4.2 B-C). In contrast, forebrain-like populations expressed SOX1 and OTX2, and lacked GFP expression (Figure 4.2 E-F) which was found in neighboring retinal populations.
Fig. 4.2. Emergence of distinct retinal and forebrain regions from a common progenitor pool. Within 3 weeks of differentiation, plated colonies of hPSCs gradually adopted either retinal or forebrain phenotypes, as identified by bright field images (A, D). A cell line with a retinal lineage GFP reporter (SIX6-GFP) was used to identify regions of presumptive retinal populations. hPSCs expressed retinal markers CHX10 and LHX2 which localized with GFP expression and displayed a more 3-dimensional appearance (B, C). To the contrary, forebrain cells expressed SOX1 and OTX2, and remained isolated from retinal-GFP populations (E, F).
4.3.3 hPSCs organize into optic vesicle & forebrain organoids

Following this early distinction between retinal and forebrain populations, hPSCs were found to self-organize into distinct organoids populations after a month of differentiation. While retinal organoids displayed a light-outer ring around their periphery and possessed an organized, three-dimensional cup-like appearance, forebrain organoids were larger and darker in appearance (Figure 4.3 A, D).

Such morphological cues enable the easy identification and enrichment of retinal organoids apart from other neural populations. Additionally, GFP expression remained exclusive to retinal organoids, and these cells expressed retinal progenitor markers CHX10 and LHX2 in a ring-like fashion (Figure 4.3 B, C). Conversely, forebrain organoids lacked GFP expression but expressed anterior neural markers such as SOX1 and OTX2 (Figure 4.3 E, F).

4.3.4 hPSC-derived retinal organoids recapitulate the patterning and organization of the retina.

While previous hPSC-differentiation approaches have been successful in generating all the major cell types of the retina, these retinal cells develop stochastically among other neural populations and lack the organization and spatial patterning of the retina [16–18, 24]. Thus, these cells cannot accurately recapitulate the cell-cell interaction which is critical to the functioning of the retina, limiting the use of hPSCs for future translational purposes. Therefore, current efforts were directed to generate retinal cells that can better recapitulate the organization and functioning of the retina.

To do so, hPSC-derived cells were directed into cells analogous to the stages of retinal development, mainly the eye field stage, optic vesicle and optic cup stages of development. Following optic vesicle specification spearheaded by CHX10 expression (Figure 4.4), cells were directed to organize into layered, three dimensional retinal
Fig. 4.3. hPSC-derived colonies organize into distinct optic vesicle and forebrain organoids. Within one month of differentiation, two distinct organoid populations could be identified, with their retinal lineage identified by the presence of a light outer ring around their periphery (A, D). Retinal organoids were found to be organized into vesicle-like structures and exhibited CHX10 and LHX2 in conjunction with GFP expression (B, C). Conversely, forebrain organoids were loosely organized and their lack of GFP was correlated with the expression of forebrain markers including SOX1 and OTX2 (E, F).
Fig. 4.4. hPSC-derived organoids recapitulate the organization of the developing optic cup. At early stages of differentiation, hPSC-derived organoids expressed CHX10 throughout nearly all cells, indicative of their retinal progenitor state, but lacked the expression of more differentiated markers (A). Within 50 days of differentiation, hPSC-derived optic vesicles gradually expressed the retinal ganglion cell markers BRN3 and ISLET1 towards the inner layers of the organoids (B). Further differentiation yielded increased expression of photoreceptor markers such as Recoverin towards the outer layers of the organoids (C).
organoids reminiscent of the optic cup. At early stages of differentiation, hPSC-derived retinal organoids expressed CHX10 throughout nearly all cells, indicative of their retinal progenitor state, but lacked the expression of more differentiated markers of ganglion cells (BRN3, ISLET1) and photoreceptors (RECOVERIN) (Figure 4.4 A). As differentiation progressed, hPSC-derived retinal organoids were directed to mimic the birth order of retinal neurons, where ganglion cells were the earliest neurons to be specified, as demonstrated by the expression of BRN3 and ISLET1 within the basal layers of the organoids at 50 days of differentiation (Figure 4.4 B). At this stage, photoreceptors were just starting to be specified, with increasing expression of photoreceptor marker RECOVERIN by 70 days of differentiation (Figure 4.4 C). Importantly, retinal cells self-organized into appropriate inner and outer retinal layers within the organoids, with ganglions cells and photoreceptors occupying their strategic basal and apical positions, indicative of the optic cup in the retina.

### 4.3.5 Differentiation and characterization of hPSC-derived RGCs

Overall, differentiation of hPSCs over the last several years have assisted in the studies of retinogenesis, including the generation of nearly all cell types of the retina [16, 18, 20, 24]. However, protocols over the last decade have primarily focused on the differentiation and characterization of photoreceptors and RPE [16, 18, 21, 31, 32, 53, 58, 60, 81, 88], while characterization of ganglion cells remains limited. To address this shortcoming, hPSCs were directed to generate retinal organoids and analyzed for the expression of molecular and morphological markers typical of ganglion cells [51]. hPSCs could be directed into RGCs in a reliable manner, and 36.1 ± 1.7% cells expressed BRN3 within 40 days of differentiation, indicative of retinal ganglion cells (Figure 4.5 A). These cells exhibited a variety of molecular markers associated with ganglion cells, including PAX6, ISLET1, HUC/D and RBPMS (Figure 4.5 B-E) and extended MAP2 positive neurites typical of RGCs (Figure 4.5 F).
Fig. 4.5. Derivation and characterization of RGCs from hPSCs [51]. 35% of retinal progenitor cells acquired RGC-specific BRN3 expression after 40 days of differentiation (A). hPSC-derived BRN3-positive cells expressed a cohort of RGC-related markers, including PAX6, ISLET-1, Hu C/D and RBPMS (B-E) and extended MAP2-positive neurites (F). Upon further maturation, cells displayed elaborate RGC-like morphologies, including cytoskeletal compartmentalization of MAP2 and TAU expression (H). Expression of RGC-specific markers was demonstrated to increase via qRT-PCR increased as cells transitioned from a retinal progenitor state (Day 25) to a RGC phenotype (Day 50) (I), and RGC-specific gene expression remained exclusive to the retinal populations (J). Significant differences indicated as ( * = p < 0.05, ** = p < 0.01, *** = p < 0.005, **** = p < .001).
Interestingly, the expression of MELANOPSIN among a small subset of neurons indicated the presence of intrinsically photosensitive retinal ganglion cells (ipRGCs) (Figure 4.5 G). Upon further differentiation, RGCs extended lengthy neurites, and exhibited compartmentalization of somatosensory MAP2 expression and axonal Tau expression (Figure 4.5 H), indicating the ability of hPSC-derived RGCs to mature in vitro. Furthermore, the progressive acquisition of a RGC identity from a retinal progenitor pool was characterized using quantitative RT-PCR techniques, where expression of RGC-related genes significantly increased over 50 days of differentiation (Figure 4.5 I). As expected, the upregulation of RGC markers remained exclusive to the hPSC-derived retinal populations, when compared to age-matched population of non-retinal cells (Figure 4.5 J).

4.3.6 Modeling glaucomatous degeneration with hPSC-derived retinal organoids.

hPSCs can be generated from any somatic cell of the human body, and therefore, they serve as a unique tool to study and model diseases in vitro [3, 5]. With the ability to derive and characterize RGCs from hPSCs, such cells can be employed for future translational applications, including disease modeling, as well as drug screening purposes. As such, current efforts were focused towards the use of hPSC-derived RGCs for modeling glaucoma, which is primarily characterized by the loss of retinal ganglion cells (RGCs). As projection neurons of the retina, RGCs effectively serve as the connection between the eye and the brain and therefore, loss of ganglion cells in optic neuropathies such as glaucoma leads to blindness [73, 133, 207]. Therefore, to model RGC degeneration in glaucoma, fibroblasts were isolated from a patient who had an inherited form of glaucoma (E50K mutation in the Optineurin gene) [208, 209] and were reprogrammed into hPSCs. Preliminary experiments in the lab demonstrated that patient-derived RGCs were highly susceptible to death by apoptosis [51]. Therefore, this phenotype was further investigated using three-dimensional methods.
of differentiation, with the goal to model the effects of retinal ganglion cell degeneration on the retinal microenvironment and disease etiology.

Similar to control cells, patient-derived hPSCs were directed to generate retinal organoids, that can mimic the timings and organization of the retina. As such, no differences were observed in the ability of patient-derived hPSCs to differentiate into RGCs and occupy the basal layers of the organoids. Following RGC differentiation, control and patient-hPSCs were analyzed for expression of activated caspase-3 within the inner and outer layers of the retinal organoids, where BRN3 expression was representative of inner retinal RGCs. Patient-derived hPSCs exhibited significantly elevated caspase-3 activation within retinal organoids, indicative of apoptosis as a possible mechanism for glaucomatous neurodegeneration (Figure 4.6 A-B). Interestingly, activated caspase-3 expression was significantly expressed with the inner layers of the patient-derived organoids when compared to the outer layers of the retina (Figure 4.6 C-D). As such, the use of patient-derived organoids has helped to demonstrate that degeneration in glaucoma is specific to the RGC layer, and remains distinct from outer retinal neurons within the organoids (Figure 4.6 E).

4.4 Discussion

The recent advent of three-dimensional methods of differentiation have revolutionized the use of hPSCs for studies of the retina. While several groups have demonstrated the derivation of retinal organoids [21,27–29,32,34], the events that lead to the specification of these structures from hPSCs remained unclear. In this study, we describe the stepwise acquisition of a retinal phenotype from an unspecified pluripotent population. Our data demonstrates the subsequent differentiation of hPSCs into the eye field, optic vesicle and optic cup stages of development, leading to the enrichment of retinal organoids from other neural populations (Figure 4.1- 4.3). These retinal organoids retain a laminated structure, with the demarcation of inner and outer
Fig. 4.6. hPSCs were established from a patient with an E50K mutation associated with normal-tension glaucoma. Patient-derived retinal organoids exhibited significantly elevated caspase activation, indicating increased susceptibility of patient-RGCs to apoptosis (A-D). Interestingly, caspase upregulation remained confined to inner layers of these organoids (E), demonstrating the ability of retinal organoids to effectively model RGC degeneration in optic neuropathies. Significant differences indicated as ( * = p < 0.05, ** = p < 0.01, *** = p < 0.005, **** = p < 0.001).
retinal cells evident as early as 50 days of differentiation (Figure 4.4). Therefore, hPSC-derived retinal organoids have emerged as a robust model for studies of retinogenesis, where the resultant retinal neurons possess the phenotypic and hierarchical organization typical of the retina.

While our data demonstrates the ability of organoids to model preliminary stages of retinogenesis, earlier stochastic differentiation methods have also been utilized for the derivation of specific retinal cell types [16, 18, 20]. In particular, research over the last decade has focused on the differentiation of hPSCs into RPE and photoreceptors [18, 19, 57, 80, 81, 88, 135, 139, 189], with the description of photoreceptor maturation within organoids only recently described [29, 35]. This has not only enabled the establishment of novel and exciting approaches to studying human retinogenesis in vitro, but has also paved the way for translational application of these retinal cells. Furthermore, the ability to derive hPSCs from patients allows for subsequent use in modeling retinal degenerative diseases, with these approaches demonstrated to be successful for diseases that affect the outer retinal cells including AMD, BEST disease, Retinitis pigmentosa [11, 15, 36, 59, 90, 102, 104–106, 108, 204], leading to the possibility of developing potential therapeutic strategies to combat the degeneration.

However, disease modeling efforts with hPSCs remain limited for inner retinal neurons like RGCs. This limitation mostly stems from the lack of appropriate markers to efficiently generate and uniquely characterize inner retinal neurons among differentiation cultures of hPSCs [16, 19, 49]. Additionally, the lack of other interactions and signaling from other components of the eye may limit RGC development in vitro, leading to the death of these cells in long term cultures of hPSCs [29, 35]. We have recently demonstrated RGC differentiation from hPSCs at 36.1 ± 1.7% efficiency, with the reliable identification of RGC cells among differentiating retinal populations facilitated by the stepwise differentiation of these cells through a retinal organoid intermediary [51]. To better attribute retinal characteristics to RGCs, the current study
utilized enhancements to three-dimensional methods of differentiation. The resultant
retinal organoids mimicked the architecture of the retina, where RGC differentiation
followed temporal and spatial cues, leading to their preliminary characterization
within the basal layers of the organoids (Figure 4.4). Overall, the results of this study
demonstrate the potential use of these differentiation approaches for phenotypic and
functional characterization of RGCs and their subtypes, with the potential use of
organoids for modeling RGC degeneration in optic neuropathies. To further explore
these abilities, efforts were undertaken to utilize hPSC-derived retinal organoids for
disease modeling of the inner retina.

Degeneration of RGCs in optic neuropathies like glaucoma severs the visual con-
nexion to the brain, leading to progressive loss of vision and eventual blindness.
While retinal organoids have enabled studies of differentiation and development of
RGCs [21,27–29,35], modeling glaucomatous neurodegeneration with hPSCs is chal-
lenging. Glaucoma is most commonly caused by increased intraocular pressure in the
eye, which is tricky to model in vitro. Moreover, the disease phenotype is primarily
casted due to the degeneration of RGC axons in the optic nerve head, and current
efforts to model glaucoma are limited in their ability to model this aspect of axonal
neurodegeneration. Even with the successful use of retinal organoids for studies of
retinogenesis, these structures lack an exit route for RGC axons, which typically con-
nect with their post-synaptic targets in the brain.

Therefore, such hPSC-derived RGCs lack the axonal fasciculation that forms the op-
tic nerve, limiting their functional maturation and their use as models for loss-of
function studies in optic neuropathies. In spite of these challenges, studies of the
disease mechanisms in early stages of glaucoma will help to understand the disease
pathophysiology that results in RGC degeneration. For this purpose, genetic models
of glaucoma can help to provide insight into potential mechanisms of glaucomatous
neurodegeneration that begin in the cell bodies of RGCs, and can uncover the mech-
anisms that lead to axonal death and subsequent loss of vision in glaucoma [210,211]. For this purpose, hPSCs were generated from patients with a genetic disposition to glaucoma, with a particular focus on mutations that directly affect RGCs. The E50K mutation in the optineurin gene satisfies these criteria [208,209,212,213], and recent efforts have focused in characterizing the disease phenotype using hPSCs. As such, care was taken to ensure that the disease phenotype is only seen in the affected cell type, and remains absent from other unrelated cell types. We have recently demonstrated the differentiation of RGCs from patient-derived hPSCs, and elevated markers of apoptosis were only seen in patient-derived RGCs and remained absent in the control cells [51]. With the presentation of the disease phenotype, such patient-RGCs can be used as a testing platform for screening potential neuroprotective factors that can rescue the disease phenotype.

To further demonstrate the effect of glaucomatous neurodegeneration within the retinal tissue, patient-derived hPSCs were directed to differentiate into retinal organoids (Figure 4.6). So far, our results demonstrate no differences in the ability of patient-derived hPSCs to differentiate into RGCs within the basal layers of the organoids. However, patient-derived cells demonstrated increased activation of apoptotic markers, indicating apoptosis as the potential mechanism for RGC degeneration in patient samples. Importantly, activation of caspase expression remained exclusive to the RGCs layer and remained absent from other retinal layers. This is the first demonstration of RGC layer-specific degeneration within organoids, demonstrating the ability of organoids to study the effects of neurodegeneration within the retinal microenvironment.

Overall, this study represents one of the many strategies undertaken to advance the use of retinal organoids for translational studies of the retina. As such, increased reproducibility of retinal organoid generation, detailed characterization of retinal neurons and their circuitry will assist in development of better organoid systems for the
hPSC-based disease modeling efforts. As present, retinal organoids are better suited for modeling photoreceptor diseases, whose development and maturation within these organoids has been well documented [21,22,29,34–36,135]. Their development in the outer layers of the organoids facilitates easy access for loss-of-function studies in diseases like retinitis pigmentosa. Subsequently, such hPSC-based mini-retinas can assist in drug-screening efforts [21,32,35,36,51], with the goal to identify candidate factors to alleviate the neurodegeneration of retinal neurons. In the absence of RGC axonal networks within these organoids, current readouts for drug screening efforts are limited to increased survival of the cells. Future efforts will need to focus on RGC axonal development within retinal organoids, with the eventual goal to rescue degeneration and loss of vision in glaucoma. Ultimately, such hPSC-based disease modeling approaches will help to gain insights into early disease mechanisms, and help identify candidate factors that rescue neuronal degeneration and progressive loss of vision in retinal diseases.
5. DISCUSSION

Over the last decade, hPSCs have emerged as powerful tools for studies of human development and disease, as they can self-renew indefinitely and generate all the cell types of the body. While initial efforts to derive hPSCs relied on embryonic sources, the advent of induced pluripotent stem cells has allowed for the ability to generate hPSCs from somatic cells of the body, including skin, blood or urine [3, 5, 28, 214]. When derived from patients, hPSCs can serve as a renewable supply of patient cells for modeling diseases and potential cell replacements, making them popular models for future translational applications [77, 83, 98, 100, 203, 215].

While hPSCs can be used for studies of any cell type of the body, such approaches were directed at retinal diseases for several reasons. Firstly, retinal degenerative diseases affect millions of people worldwide, causing debilitating vision loss and eventual blindness, and remain incurable to date [91, 216, 217]. Secondly, the retina is relatively simple in nature compared to other regions of the central nervous system, and its surgical accessibility and immune-privileged location make it amenable to cell transplants and drug candidates, the effects of which can be monitored using non-invasive technologies [112]. Finally, hPSCs provide access to some of the earliest events of human development, making them ideal candidates for studies of early events of human retinogenesis.

Therefore, initial efforts were focused on optimizing the differentiation of hPSCs to generate retinal cells in a reliable manner, with the goal to utilize such in vitro retinal cells for regenerative purposes. Since events of human retinogenesis in situ remain inaccessible to investigation, initial hPSC differentiation protocols were developed based on the plethora of information on developmental studies in animal models, primary
cell culture lines and limited analysis of human fetal retina [218–221]. As the first step of vertebrate retinogenesis is the establishment of the anterior neuroepithelium, hPSCs were first directed to a neural phenotype using a combination of endogenous or exogenous factors that modulate signaling pathways within these cells. After this stage, hPSCs can be coaxed to spontaneously generate retinal cells, or be directed to do so in a stepwise manner. The latter approach allowed for the enrichment of optic-vesicle like structures, that could be dissociated to allow for the differentiation and maturation of retinal cells on an ECM matrix (laminin/matrigel). With this approach, all the major cell types of the retina could be generated in a temporal fashion, with ganglion cells as the earliest neurons to be specified, while bipolar cells/Muller glia are the last. However, differentiation of retinal cells on an ECM matrix prevented the cells from forming three-dimensional structures typical of the retina, and limited their functional maturation.

The field has now shifted to a three-dimensional method of differentiation, where hPSCs can be directed to self-organize into discrete optic vesicle-like structures that could be enriched from their forebrain counterparts [21,22,28]. Further characterization demonstrated that the ability of organoids to self-assemble into a pseudostratified neuroepithelium, where retinal cells were arranged in a multilayered structure. As a result, retinal cells retained close cell-cell contact association, assisting in the synchronous differentiation and spatial patterning of these cells into a precise apical-basal structure. The stepwise differentiation of these organoids has been documented by several groups, where retinal progenitors in organoids sequentially differentiate into a basal RGC layer, an intermediate layer of interneurons and an outer layer of photoreceptors [27–29,31,32,35,36]. Additionally, the use of three-dimensional methods of differentiation has been shown to assist in enhanced differentiation and maturation of outer retinal neurons, where cells possessed characteristic morphological, molecular as well as rudimentary functional properties typical of photoreceptors [29,35].
Overall, the ability of hPSCs to accurately recapitulate events of human retinogenesis illustrates their potential in generating bona fide retinal cell types of future clinical application of these cells. Moreover, the derivation of hPSCs from somatic cells of patients constitutes a powerful and unique approach for individualized disease modeling approaches and development of personalized treatments for retinal degenerative diseases. While hPSCs are limited in their ability to model all the direct and indirect manifestations of a disease, these cells can effectively recapitulate hallmark features and provide insights into disease mechanism and its pathophysiology [222]. This ability of hPSCs can be best illustrated by the multitude of studies related to modeling RPE degeneration in diseases like Age-related macular degeneration (AMD), Best disease and Gyrate atrophy, to name a few [21, 59, 87, 102, 105, 108, 168, 223]. RPE degeneration in these diseases severely impacts the health and integrity of photoreceptors, and affects the ability of the retina to function as a homeostatic unit for processing visual information. Moreover, a large majority of retinal degenerative diseases remain incurable to date and current models are limited in their ability to fully recapitulate both early and end-states of disease. For example, the absence of a macula in mouse and cell-based models of diseases like AMD limits the ability to model RPE degeneration, and presents significant roadblocks in understanding disease etiology [224, 225].

Nevertheless, patient-derived hPSCs have been successful in providing valuable insights into early stages of RPE degeneration in diseases. Additionally, these cells can serve as platforms for testing and developing drug-based therapies, with the goal to combat the degeneration and rescue loss of vision in retinal diseases. For this purpose, several protocols have demonstrated the derivation of RPE either by spontaneous differentiation [52, 56, 61, 121], or stepwise protocols [16, 18, 20, 24, 54, 88], and these cells exhibit a characteristic hexagonal morphology, pigmentation, distinct apical-basal junctions and some functional characteristics, including phagocytosis and growth factor secretion [53, 58, 167]. Importantly, hPSCs derived from AMD and Best disease
patients demonstrate hallmark features of the RPE dysfunction when compared to control cells, and have helped to uncover disease pathophysiology and potential mechanisms of neurodegeneration [59, 108, 128, 204, 223]. As such, these studies have also helped evaluate risk-alleles for diseases, and establish links between the genotype and the disease pathology [6, 56, 101, 104, 108, 109, 136, 205].

In addition to disease modeling, hPSC-derived RPE can also serve as a platform for testing candidate drugs and investigate critical time points for medical intervention. However, such strategies cannot be employed in advanced diseases states, where multiple retinal cell types and their connections are lost. In these cases, the patient derived RPE can be transplanted into the degenerating retina, with the goal to replace the damaged cells with their function counterparts. For this purpose, hPSC-derived RPE were transplanted into Royal College of Surgeons rats, and these cells demonstrated integration within the host retina [129, 226, 227] and increased long-term survival of the host photoreceptor cells [56, 116, 121]. The success of these experiments led to the utilization of hPSC-derived RPE for in clinical trials in patients with AMD and Stargardt’s disease, where patients demonstrated increased visual acuity 6-12 months post transplantation [14, 130], and such efforts are currently being utilized for several clinical trials worldwide [128, 228]. Additionally, recent strategies have focused on three-dimensional models of transplantation, where RPE can either be transplanted as preformed sheet or as a part of a scaffold system [123, 126, 229, 230], enabling better integration and survival within the retina.

While replacement of RPE may be sufficient for partial functional restoration, diseases like Leber’s Congenital Amaurosis and retinitis pigmentosa may require additional transplants of functional photoreceptors to rescue the visual impairment. For this purpose, a variety of endogenous and exogenous signaling cues have been extensively described to direct the differentiation of hPSCs into photoreceptors [20, 21, 23, 25, 49]. When generated from patient-derived hPSCs, the resultant photoreceptors can be
utilized for disease modeling as well as cell replacement purposes. hPSC-derived RPE possess characteristic hexagonal morphology, pigmentation and grow in distinctive patches that enable their easy identification within bright field cultures, and can then be manually isolated and expanded in vitro. However, photoreceptors develop among other retinal neurons and lack such distinctive morphological features that enable the unique derivation of photoreceptors apart from other retinal populations (Wright et al., 2014). Additionally, differentiation of functional photoreceptors is time consuming, and multiple subtypes may develop within a heterogenous populations of retinal cells. Until recently, photoreceptor maturation could only be achieved upon in vivo transplantation or co-culture experiments, but the recent description of three-dimensional retinal organoids has enabled the morphological and rudimentary functional maturation of these cells in vitro [28,29,31,35].

Therefore, advancements in photoreceptor derivation coupled with patient-derived hPSCs have enabled the ability to model photoreceptor degeneration in vitro. hPSC-derived photoreceptors from genetically inherited forms of retinitis pigmentosa demonstrated reduced cell survival, which were then used as a platform for testing pharmacological intervention approaches [11,69,90]. Such hPSC-based approaches have enabled analysis of disease pathophysiology in greater detail, and provide a customized platform for testing patient-specific therapy strategies [21,78,101,103–105]. In addition to disease modeling approaches, hPSC-derived photoreceptors can assist in cell replacement strategies, with the expectation that the successful integration of these cells will enable their reconnections with the bipolar and horizontal cells, enabling the potential repair of the retinal visual circuitry. For this purpose, several groups have demonstrated the transplantation of hPSC-derived cells in animal models of disease, leading to successful survival and integration of these cells within the host retina and improved light response, [8,10,15,49,56,86,114,116,121,123,231], with the eventual goal to restore the functionality of these cells. However, recent studies have warranted further investigation in such transplantation experiments, as evidence
suggests that majority of donor cells exchange cytoplasmic material with host cells instead of integration [232–234]. While this calls for a potential reconsideration of previous cell replacement approaches, it also represents a novel way for cell-based treatment approaches in retinopathies, and future efforts will need to focus on better understanding of the transplantation and the cell integration process.

While hPSCs have promoted the understanding of disease pathophysiology of outer retinal diseases, such efforts have been largely limited for the inner retinal neurons, such as retinal ganglion cells (RGCs). The loss of RGCs is characteristic of several optic neuropathies, including glaucoma, and affects over 60 million people worldwide. While current treatments for glaucoma have mostly focused on lowering of intraocular pressure (IOP) in the eye, this approach does not stop disease progression, and is ineffective in normal tension glaucoma cases, where IOP is not an issue. The multifactorial causes for glaucoma, and the added complication of restoring the long axonal connections to the brain have complicated efforts to restore the functionality of these cells in optic neuropathies [133]. Despite these shortcomings, recent efforts have focused on detailed characterization and reliable identification of RGCs within differentiating cultures of hPSCs [63, 65–68, 93], enabling the use of these cells for downstream translational applications. As such, the recent derivation of hPSCs from patients with genetic determinants of glaucoma has assisted in modeling the hallmark features of RGC degeneration in vitro [51, 94].

While patient-derived cells have enabled identification of potential neuroprotective agents to combat the disease phenotype, these approaches are likely ineffective for later stages of diseases, where severe RGC axonal degeneration causes progressive loss of vision. Initial cell replacement efforts with mouse-PSCs cells and primary cell lines have demonstrated initial integration within the retina [235, 236], but the lack of axonal outgrowth and synaptic connections have led to no functional improvement following transplantation [228]. Still, the ability of transplanted RGCs to survive and
integrate represents important implications for the use of these cells for clinical applications. Additionally, the use of three-dimensional models of differentiation, coupled with better axonal characterization of RGCs will likely facilitate development of therapies for RGC replacement in optic neuropathies.

Overall, hPSCs represent a pivotal and unique model for studies of retinal development, and the knowledge gained from developmental studies has enabled the stepwise derivation of retinal cells in a developmentally appropriate manner. While hPSC-derived retinal cells have been demonstrated to aid in a variety of translational purposes, including disease modeling, drug screening and cell replacement, a large majority of retinal degenerative disease remain incurable to date. Therefore, the work described in this dissertation focuses on optimizing the various steps involved in derivation of hPSCs, their maintenance as well as retinal differentiation approaches, with the goal of facilitating better translational applications of hPSC-derived retinal cells into the clinic.

One of the biggest advantages of hPSCs is their ability to be derived from somatic cells of patients, which provides renewable access to patients genetics, enables studies of disease modeling, and generates autologous cell types for transplantation purposes. The ability to generate human induced pluripotent stem cells was first described in 2007, where researchers utilized retroviral vectors to deliver a cocktail of genes to skin cells and reprogram them to a pluripotent cell fate [3,5]. However, retroviruses are not an ideal option for generating hPSCs from adult tissues, as they only infect actively dividing cells, which constitute a minority population in adult sources of somatic cells. Additionally, the use of viruses introduces risks of genomic integration, insertional mutagenesis and tumors due to ectopic expression of transgenes [223,237,238]. Since then, advances in reprogramming technology have led to the development of alternative methods of reprogramming, which are safer and more efficient than the original viral vectors. Additionally, non-integrating viral systems such as adeno-associated or
Sendai viruses have also been developed, and the choice of the reprogramming method depends on downstream applications of these cells [172, 182, 183, 192]. For example, Sendai viruses cannot be yet used for clinical studies. However, episomal vectors can be generated in a GMP-compliant manner and have been utilized to reprogram a variety of somatic cell types.

Since initial retinal differentiation protocols mostly utilized virally-reprogrammed cell lines, efforts were undertaken to test the choice of reprogramming method on the ability to derive retinal cells. For this purpose, retinal differentiation of retroviral lines was compared to lines of hPSCs derived through non-viral mRNA-based methods [26]. As an alternative to viral based reprogramming methods, mRNA-based reprogramming techniques represents an elegant, non-integrating approach to generate pluripotent cells [149]. These approaches involve a transient mode of transfection, and do not require any additional clean-up of residual vectors after the reprogramming event. While reprogramming through this method is time intensive and involves repeated transfections over two weeks, the efficiency of mRNA reprogramming is relatively high when compared to other reprogramming methods [239]. Since most hPSC-based retinal differentiation protocols were initially developed using virally reprogrammed cell lines, efforts in the Meyer lab focused on analyzing the effect of reprogramming on differentiation of retinal cells.

For this purpose, three retroviral (riPS) and three mRNA reprogrammed lines (miPS) were simultaneously derived from fibroblasts and comparisons were made at various stages of differentiation. As expected, mRNA reprogramming was more efficient when compared to retroviral methods, but no overt differences were observed in acquisition of a retinal phenotype. Importantly, the use of miPS lines eliminated the risks of genomic insertion and mutagenesis associated with other methods of reprogramming, and the resultant cells could faithfully generate all the major retinal cell types which are better suited for future clinical applications. Following reprogramming of hPSCs,
the choice of culture conditions and differentiation methods affects their downstream applications for translational studies of the retina. hPSCs have been traditionally grown on inactivated mouse embryonic fibroblasts (MEF), and the cells are usually supplemented with serum at some point during the differentiation. However, the use of animal products or undefined components in the culture conditions introduces risks of zoonosis, compromising the immunogenic profile of these cells and increasing risks of graft rejection when used for future cell replacement purposes. Even the use of undefined extracellular matrix proteins such as Matrigel or laminin promote variability among batches, limiting the development of reproducible and clinically translatable retinal cells.

Most of the earliest retinal differentiation protocols utilized MEF or serum at some point during differentiation [16,18–20], particularly in the early maintenance of pluripotency, as well as initial stages of differentiation. While such approaches have enabled the successful generation of retinal cell types, the ability to successfully derive retinal cells in the absence of animal products remained to be described. Furthermore, while some groups have described the early maintenance of hPSCs in xeno-free conditions [150–152], serum was still included at later stages of neural and retinal cell types. Therefore, we described a xeno-free approach for hPSC differentiation utilizing a chemically defined system that eliminated the need for any animal products [26,90]). With this approach, hPSCs could maintain their pluripotent state and could be directed to generate retinal cells in a stepwise manner, similar to traditionally grown hPSCs on feeder cells or Matrigel. Importantly, all the major retinal cell types could be derived with a xeno-free approach [26], including ganglion cells, photoreceptors and RPE, with the latter currently being employed in clinical trials.

While we did not observe any significant differences in the efficiency of retinal specification across a MEF, matrigel or xeno-free system, the use of a completely xeno-free system enabled enhanced differentiation and maturation of hPSC-derived ventral
brain neurons upon transplantation into rodent models of Parkinsons disease [240]. Such studies further demonstrate the suitability of xeno-free approaches for hPSC-based differentiation approaches, and provide the potential for such chemically defined systems to improve the reproducibility and functionality of hPSC-derived cells for downstream clinical applications.

With the development of refined techniques for generating and maintenance of hPSCs, several modifications have also been made to existing retinal differentiation approaches to facilitate generation of quality and reproducible retinal cells. The earliest hPSC differentiation protocols primarily utilized a variety of exogenous factor to direct cells to a neural and subsequently a retinal fate, demonstrating the preliminary characterization of hPSC-derived retinal cell [16, 18, 24, 49]. However, some of these approaches led to accelerated differentiation of retinal cells among heterologous populations of differentiating hPSCs, limiting the ability to faithfully recapitulate the timing and events of human retinogenesis.

An alternative to this approach was described in 2009, where hPSCs were directed to adopt a default neural fate, and retinal fate was acquired in a stepwise manner [20]. Thus, this approach eliminated the need for exogenous signaling factors to direct differentiation of hPSCs, and the resultant retinal cells could successfully recapitulate the timing of retinogenesis. While such approaches allowed for the generation of all the major retinal cell types, these cells failed to acquire a mature morphological and functional phenotype even after four months of differentiation. Therefore, recent studies have demonstrated the generation of hPSC-derived retinal organoids in a manner that effectively recapitulates the patterning and organization during retinogenesis [21,27–29,31,34,35,85,105,241]. With this approach, hPSCs can self-assemble into a multilayered retinal structure, allowing for extended differentiation and maturation of retinal cell types in vitro.
Therefore, advances in hPSC differentiation methods over the last several years have led to the generation of all the major retinal cell types in a developmentally appropriate manner. To utilize hPSCs-derived retinal cells for disease modeling and cell replacement purposes, these cells will need to be appropriately characterized and assessed for functionality. The characteristic hexagonal morphology and pigmentation of RPE has enabled their ease of identification, isolation and maturation among differentiating cultures of hPSCs, and they have been extensively utilized for disease modeling as well as cell replacement studies of the retina [10,11,21,59,81,88,89,102,108]. Photoreceptor cells possess unique molecular markers and a distinct functional response, and therefore these cells have been widely studied with hPSCs [8,11,49,83,90,102,103,134,242], including the recent description of their maturation within retinal organoids [29,35].

Outside of RPE and photoreceptors however, a large majority of the retinal neurons cells lack easily discernible morphological or phenotypic characteristics, and characterization of inner retinal neurons such as RGCs has been mostly overlooked. Therefore, we described a step-wise approach to document RGC development and maturation from hPSCs, where a retinal progenitor intermediate was used to reliably identify and characterize the ensuing BRN3-positive RGCs [51]. Additionally, the timing and spatial organization of RGCs could be effectively visualized using retinal organoids, illustrating the applicability of retinal organoids for studies of RGC maturation and axon guidance.

Thus, refinements in generation, phenotypic and functional characterization of hPSC-derived retinal cells has enabled the ability of patient-derived hPSCs to assist in modeling complex retinal disorders. While not all aspects of retinal diseases are amenable to in vitro disease modeling approaches, hPSCs are particularly suited for modeling genetic determinants of these diseases that directly affect a specific retinal cell type. As such, the detailed characterization of hPSC-derived retinal cells has enabled
morphological, phenotypic and well as functional comparisons between control and patient-derived hPSCs. These patient-derived hPSCs not only help to bridge the gap between the precise gene mutation and their biological underpinnings, but also help reveal previously unknown genetic modifications that can contribute to the disease phenotype, which can then be utilized as testing platform for development of novel therapeutic strategies.

However, the ability to generate hPSCs from patients does not guarantee the presentation of a disease phenotype. One probable reason is that reprogramming of hPSCs erases all adult age-related markers and resets cells to an embryonic stage of development, which limits their ability to serve as effective models for late-onset diseases [226, 243]. Therefore, hPSC-derived retinal cells require months to attain mature differentiated markers, but are still functionally immature when compared to their adult retinal counterparts [29, 35]. Factoring these limitations into the study design will facilitate better translation of hPSC-based disease modeling approaches into the clinic.

So far, hPSCs are better suited for modeling early stages of monogenic disorders, especially when they affect a single retinal cell type. Such approaches have been especially successful for diseases like gyrate atrophy, retinitis pigmentosa, and Best disease [87, 89, 204, 205, 226], but are challenging for multifactorial diseases such as AMD or glaucoma. For complex retinal degenerative diseases, hPSCs can assist in modeling certain aspects of the disease state, with the goal to resolve at least one part of the complex puzzle that contributes to the disease phenotype. For example, efforts to model AMD have focused on rescuing in vitro RPE degeneration [14, 15, 104, 108], with the potential to apply these strategies to rescue RPE degeneration seen in the macula. Similarly, we utilized patient-derived RGCs to model early stages of RGC degeneration in glaucoma [51], which enabled the identification of potential neuroprotective agents that can increase the survival of these neuron. Additionally, these
results also demonstrate the utility of hPSC-based retinal organoids in recapitulating the layer-specific retinal degeneration, which was lacking in previous disease modeling approaches.

With recent advancements in reprogramming strategies, differentiation techniques as well as better characterization of retinal cells, hPSCs continue to emerge as the frontier for translational studies of the retina. The ability to generate patient-specific hPSCs allows for the potential for gene therapy, where patient-cells can be genetically corrected and autologously transplanted to “fix the defect” [125,128,244]. Currently, hPSC-derived retinal cells are being utilized in 10 clinical trials for the treatment of retinal degenerative diseases [228]. While RPE has been the most extensively studied cell type for replacement purposes, the recent success of photoreceptor derivation methods and their transplants in animal models [19,49,95,114,116,242] indicate their potential to enter clinical trials next. Further studies will need to determine if the transplanted cells can overcome the immunological challenges and effectively integrate and rewire the synaptic connections within a degenerating retina and complete the visual circuitry.

While early clinical data from RPE transplants show promise for improving visual acuity [14, 130], the successes of hPSC-derived cells in other clinical trials remain to be seen. Therefore, several strategies have employed over the last several years to facilitate better translation of hPSCs into the clinic. The use of integration-free hPSC reprogramming techniques, xeno-free approaches and GMP-compliant cell lines and pre-screening of immunocompatible recipients [25, 26, 78, 90, 239, 245, 246] can help to minimize graft rejection issues. In advanced states of diseases where loss of multiple retinal cell types has resulted in atrophy of ocular tissue, transplantation of retinal organoids facilitate the delivery of a pre-formed retinal tissue-like structure and can effectively integrate and replace a significant part of the degenerating retinal tissue [134]. Significant challenges still remain for replacements of RGCs, whose
axons need to transverse long distances and form functional synaptic connections in the brain. Current efforts have focused on further characterization of RGCs and their subtypes, and their neurite outgrowth within retinal organoids [93], with the goal to develop better three-dimensional models that can assist in developmental, clinical as well as pathological studies of retinal degenerative diseases.
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