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Robust Differentiation of mRNA-Reprogrammed Human Induced Pluripotent Stem Cells Toward a Retinal Lineage

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ABSTRACT

The derivation of human induced pluripotent stem cells (hiPSCs) from patient-specific sources has allowed for the development of novel approaches to studies of human development and disease. However, traditional methods of generating hiPSCs involve the risks of genomic integration and potential constitutive expression of pluripotency factors and often exhibit low reprogramming efficiencies. The recent description of cellular reprogramming using synthetic mRNA molecules might eliminate these shortcomings; however, the ability of mRNA-reprogrammed hiPSCs to effectively give rise to retinal cell lineages has yet to be demonstrated. Thus, efforts were undertaken to test the ability and efficiency of mRNA-reprogrammed hiPSCs to yield retinal cell types in a directed, stepwise manner. hiPSCs were generated from human fibroblasts via mRNA reprogramming, with parallel cultures of isogenic human fibroblasts reprogrammed via retroviral delivery of reprogramming factors. New lines of mRNA-reprogrammed hiPSCs were established and were subsequently differentiated into a retinal fate using established protocols in a directed, stepwise fashion. The efficiency of retinal differentiation from these lines was compared with retroviral-derived cell lines at various stages of development. On differentiation, mRNA-reprogrammed hiPSCs were capable of robust differentiation to a retinal fate, including the derivation of photoreceptors and retinal ganglion cells, at efficiencies often equal to or greater than their retroviral-derived hiPSC counterparts. Thus, given that hiPSCs derived through mRNA-based reprogramming strategies offer numerous advantages owing to the lack of genomic integration or constitutive expression of pluripotency genes, such methods likely represent a promising new approach for retinal stem cell research, in particular, those for translational applications. STEM CELLS TRANSLATIONAL MEDICINE *2016;5:417–426*

SIGNIFICANCE

In the current report, the ability to derive mRNA-reprogrammed human induced pluripotent stem cells (hiPSCs), followed by the differentiation of these cells toward a retinal lineage, including photoreceptors, retinal ganglion cells, and retinal pigment epithelium, has been demonstrated. The use of mRNA reprogramming to yield pluripotency represents a unique ability to derive pluripotent stem cells without the use of DNA vectors, ensuring the lack of genomic integration and constitutive expression. The studies reported in the present article serve to establish a more reproducible system with which to derive retinal cell types from hiPSCs through the prevention of genomic integration of delivered genes and should also eliminate the risk of constitutive expression of these genes. Such ability has important implications for the study of, and development of potential treatments for, retinal degenerative disorders and the development of novel therapeutic approaches to the treatment of these diseases.

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 [1–11]. This is particularly true as it applies to retinal differentiation of hiPSCs, for which a number of studies have demonstrated the ability to effectively use hiPSCs as a novel in vitro model of human retinogenesis [4, 12–16], including the generation of retinal-like structures from hiPSCs [13, 17–21]. Furthermore, when derived from patient samples with known genetic determinants of retinal disease, hiPSC-derived retinal cells have proved to be a promising in vitro model for studies of disease progression [13, 22–26]. Some studies have also demonstrated the potential of hiPSCs as an unlimited source for cell replacement

strategies, particularly in models of retinal degeneration [27–32].

Despite the considerable advances that have been demonstrated using hiPSCs, a number of hurdles remain before widespread use of these cells for both basic and translational retinal research. Among these difficulties includes 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 [7, 33–35], with many of these cell lines currently widely used in stem cell research [13, 14, 16, 21, 36]. Although these approaches established the proof of principle that hiPSCs could effectively be generated from somatic cells, they also possessed certain shortcomings, making them less than ideal candidates for translational applications. First, owing to the integrating nature of retroviral DNA, concerns exist regarding the integration site within the genome of the host cell [37–40]. Furthermore, these reprogramming factors were typically driven by strong constitutive promoters. Although many studies have demonstrated the silencing of these transgenes after the establishment of new lines of hiPSCs [7, 33], the potential exists for deleterious constitutive expression of these genes. Efforts have since focused on improving these methods through strategies such as excisable lentiviral vectors or nonintegrating episomal vectors [34, 41–44]. However, these still rely on DNA transfection or direct protein transduction [45, 46], which has proved difficult owing to insufficiencies in obtaining the 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 [47, 48]; however, such an approach has yet to be used 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. 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 previously demonstrated for traditional reprogramming strategies. These results were compared with retroviral-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 in this report are the first to explore the ability of mRNA-reprogrammed hiPSCs to be directed to a retinal phenotype and support the use of mRNA-reprogrammed hiPSCs as an effective and important alternative to traditional reprogramming strategies for subsequent retinal differentiation.

Materials and Methods

Reprogramming of Fibroblasts to Pluripotent State

Human foreskin fibroblast cells (BJ fibroblasts; Stemgent, Lexington, MA, http://www.stemgent.com) were maintained and expanded in media consisting of Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, minimal essential medium (MEM) nonessential amino acids, and penicillin-streptomycin. For reprogramming, approximately 50,000 fibroblasts were seeded into each well of a six-well plate. The next day, the cells were switched to Pluriton reprogramming medium (Stemgent), and mRNA reprogramming was initiated via chemical transfection of synthetic mRNAs encoding for reprogramming factors with Stemfect transfection reagent (Stemgent) beginning on day 1, with daily transfection and medium changes thereafter through day 14. To enhance reprogramming efficiency via mRNA transfection, a microRNA booster kit was similarly chemically transfected on day 0 and day 4 as per the manufacturer's instructions. 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, the medium was changed to mTeSR1 (StemCell Technologies, Vancouver, BC, Canada, http://www.stemcell.com). Typically, 5–10 colonies of newly reprogrammed iPSCs were identifiable within the first 3 weeks via both methods of reprogramming.

To establish individual lines of hiPSCs from both mRNA and retroviral reprogramming strategies, the 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. On prospective identification of successfully reprogrammed cells, individual colonies were manually isolated with a pipette and transferred to a Matrigel-coated well (BD Biosciences, Franklin Lakes, NJ, http://www.bdbiosciences.com) of a six-well plate. Isolated colonies were maintained in mTeSR1 medium and passaged as needed to expand and establish as new, individual lines of hiPSCs.

Maintenance of Undifferentiated Cells

Three mRNA-reprogrammed lines of hiPSCs (designated miPS-2, miPS-4, and miPS-6) and three retroviral-reprogrammed cell lines (designated riPS-1, riPS-2, and riPS-4) were maintained in the undifferentiated state, as previously described [14, 16, 49]. In brief, hiPSCs were maintained on six-well plates coated with Matrigel (BD Biosciences) and supplemented with mTeSR1 medium (StemCell Technologies). The medium was replaced on a daily basis, and, on reaching confluence, undifferentiated colonies were passaged using dispase (2 mg/ml; Life Technologies, Norwalk, CT, http://www.thermofisher.com). hiPSCs were typically passaged every 5 days at a ratio of 1:6.

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 [4, 12–16, 20, 25]. In brief, 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, and 2 μg/ml heparin) over the first 3 days of differentiation, as previously described [14, 16]. Embryoid bodies were plated onto six-well plates after 6 total days of differentiation with the addition of 10% fetal bovine serum to allow for attachment. The next day, the fetal bovine serum was removed, and the cells were maintained in NIM until day 16 of differentiation. At this point, cell clusters were lifted as previously described [13, 14, 16] 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 according to 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, retinal pigment epithelium (RPE) were derived from hiPSCs, as previously described [13, 14, 16].

Immunocytochemistry Analysis

Samples were analyzed by immunocytochemistry, as previously described [14, 16]. In brief, samples were collected at known stages of retinal differentiation and fixed with 4% paraformaldehyde. Primary antibodies (supplemental online Table 1) were diluted in a solution consisting of 0.1% Triton X-100, and 5% donkey serum and cells were incubated overnight at 4°C. The next day, secondary antibody was diluted and added to the cells, along with 4′,6-diamidino-2-phenylindole (DAPI) for 1 hour at room temperature. The cells were visualized and images captured using either a Leica DM5500 fluorescence microscope (Leica, Heerbrugg, Switzerland, http://www.leica.com) with a Hamamatsu Orca-R2 digital camera (Hamamatsu Phototonics, Hamamatsu City, Japan, http://www.hamamatsu.com) or a confocal/two-photon Olympus Fluoview FV-1000 MPE system (Olympus, Tokyo, Japan, http://www.olympus.com).

Data Quantification

A minimum of three samples from each cell line were plated onto poly-L-ornithine/laminin-coated coverslips, and immunocytochemistry was performed as described at the 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 2,500 cells per cell line for each marker indicated, and the number of antibody-stained nuclei was quantified using ImageJ software (NIH, Bethesda, MD, http://imagej.nih.gov/ij). Cell counts are expressed as the mean $\pm$ SE, and the percentage of cells expressing the indicated markers was compared with the total number of cells as indicated by the total DAPI-stained nuclei. To determine statistically significant differences between samples, analyses of variance were performed using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA, http://www.graphpad.com), with significant differences identified at a p value of $<.05$.

Reverse Transcription Polymerase Chain Reaction and Quantitative Reverse Transcription Polymerase Chain Reaction

Reverse transcription polymerase chain reaction (RT-PCR) and quantitative RT-PCR (qRT-PCR) were performed as previously described [13, 14, 16]. In brief, RNA was extracted using the PicoPure RNA Isolation Kit (Applied Biosystems, Foster City, CA, http://www.appliedbiosystems.com), followed by cDNA synthesis with the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, http://www.bio-rad.com). PCR amplification was performed using GoTaq qPCR Master Mix (Promega, Madison, WI, http://www.promega.com) for 35 cycles and analyzed on 2% agarose gels. For qRT-PCR analysis, cDNA was amplified with predesigned primers (β-ACTIN-Hs00969077_m1, RAX-Hs00429459_m1, CHX10-Hs01584047_m1, CRX-Hs00230899_m1) and TaqMan Universal Master Mix II (Life Technologies). For OCT4, primers were designed using the National Center for Biotechnology Information 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 mRNA expression across all cell lines. A complete list of all primer sequences is provided in supplemental online Table 2.

Results

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 [6, 7, 33–35, 43, 44, 48]. Traditionally, these genes have been delivered through retroviral methods, although newer nonintegrating 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 (Fig. 1A, 1B) to identify properly transfected/infected cells. Within the first 3 days after transfection/infection, nGFP expression was observed in nearly all fibroblasts (95.46% $\pm$ 2.81%) transfected with mRNA, and a fraction of fibroblasts in parallel cultures exhibited GFP (28.67% $\pm$ 4.14%) after infection with retrovirus (Fig. 1C, 1D), compared with untransfected cells as a negative control. Within the first 3 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 of pluripotent cells (Fig. 1E, 1F). 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 (Fig. 1G, 1H).

To establish discrete, individual lines of hiPSCs, colonies of Tra-1-60-expressing cells were manually isolated and expanded in culture, and 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. Efforts were initially undertaken to demonstrate a full complement of 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, and cell surface markers SSEA-4, Tra-1-60, and Tra-1-81 (Fig. 2A), with little variability observed between each of the newly generated lines of hiPSCs (Fig. 2B).

Beyond the expression of characteristic pluripotency factors, true pluripotent cells possess the ability to give rise to all cell types of the body. To further demonstrate the pluripotent nature of these newly generated hiPSCs, each line was allowed to spontaneously differentiate as embryoid bodies, as previously documented for newly established lines of hiPSCs [6, 7, 13, 33, 35]. After 3 weeks of growth and differentiation under these conditions, the 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 (Fig. 3).

Neuroretinal Differentiation From hiPSCs

The derivation of retinal cell types from a pluripotent cell source such as hiPSCs necessitates a stepwise progression through

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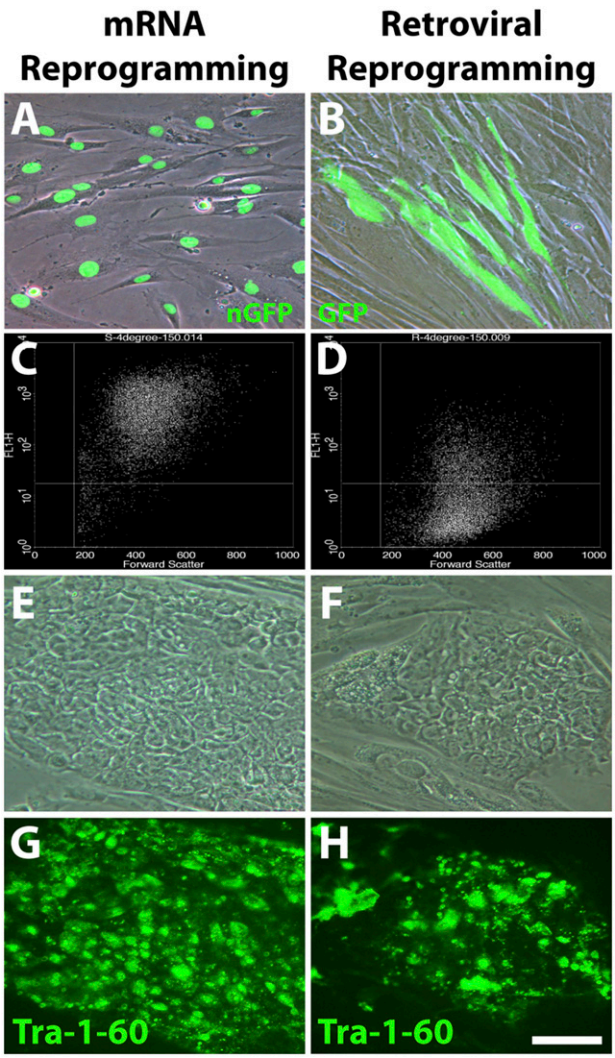

Figure 1. Reprogramming of fibroblast samples to pluripotency. Human fibroblasts were transfected with daily doses of mRNA encoding for pluripotency reprogramming factors and a nuclear GFP reporter **(A)**. Similarly, other cultures of fibroblasts were infected with retroviruses of the transcription factors, including GFP reporters **(B)**. The 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 human induced pluripotent stem cells. Scale bar = 100 μm. Abbreviations: FL1-H, intensity of green fluorescence; GFP, green fluorescent protein; nGFP, nuclear green fluorescent protein.

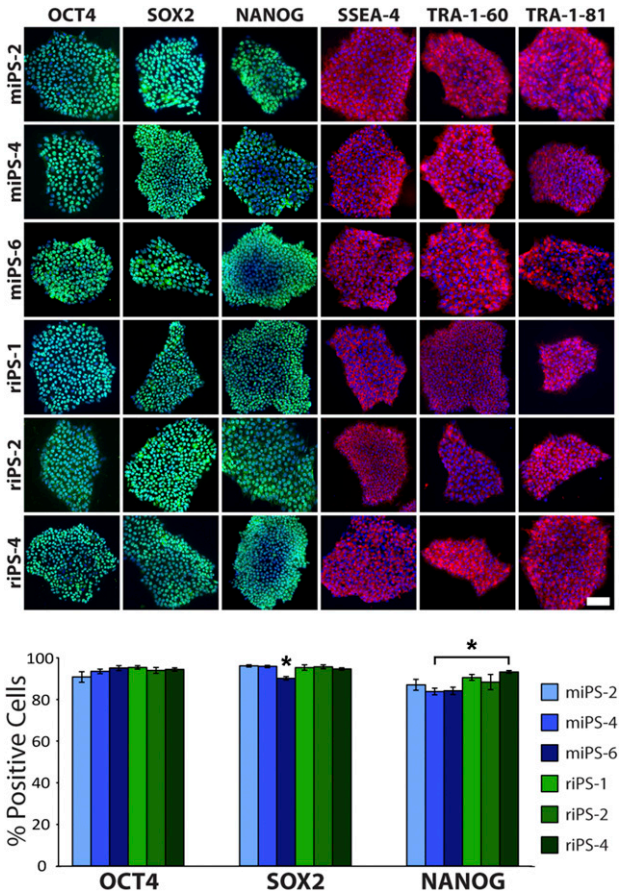

Figure 2. Establishment of lines of human induced pluripotent stem cells (hiPSCs) through mRNA- and retroviral-reprogramming methods. mRNA-reprogrammed lines of hiPSCs (miPS-2, miPS-4, and miPS-6) and retrovirally reprogrammed cell lines (riPS-1, riPS-2, and riPS-4) expressed a full complement of pluripotency-associated characteristics. Quantification of immunocytochemistry results indicated that although some lines of hiPSCs varied in the expression levels of SOX2 and NANOG, mRNA-reprogrammed hiPSCs overall did not overtly differ from their retroviral counterparts. *, $p < .05$. Scale bar = 200 μm. Abbreviations: miPS, mRNA-reprogrammed hiPSCs; riPS, retroviral-derived hiPSCs.

various stages of development, including the acquisition of an eye field phenotype, followed by the optic vesicle stage 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 also that this differentiation occurs in a manner such that each of these major stages of retinogenesis occur in a predictable and readily identifiable fashion [4, 13, 20, 21, 50]. Thus, the ability of mRNA-derived hiPSCs to differentiate in such a manner that closely recapitulates these major stages of retinogenesis was further explored.

All lines of hiPSCs derived through mRNA and retroviral reprogramming methods were directed to differentiate to an 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 (Fig. 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, compared with their retroviral-derived counterparts, and did so in a highly efficient manner.

After the acquisition of an eye field phenotype, the next major stage in retinal development is the subsequent acquisition of an optic vesicle-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 stage of retinal development within the first 30 total days of differentiation [12–14, 16, 20, 21]. These retinal progenitor cells are organized into individual OV-like structures, allowing for their subsequent isolation and enrichment. Correspondingly, miPS cells were directed to differentiate toward an optic vesicle-like stage,

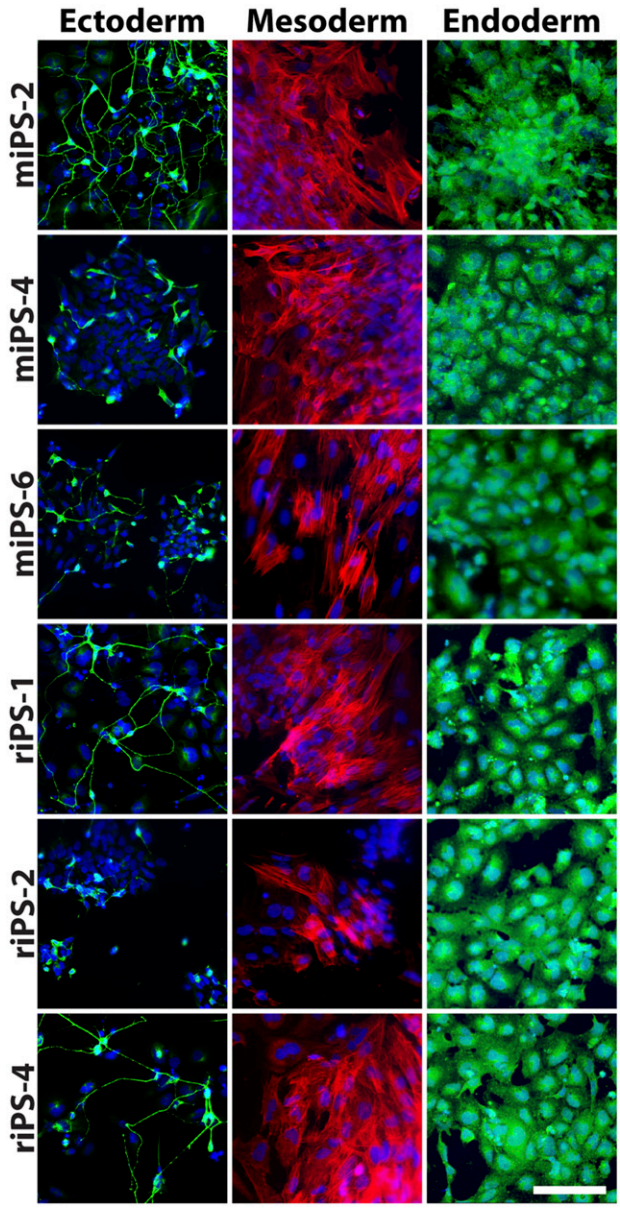

Figure 3. miPS and riPS lines generated cells of all three germ layers. To confirm the pluripotent nature of these newly generated miPS and riPS lines, differentiation was performed via the formation of embryoid bodies to further assess the pluripotent nature of these cells. All lines of human induced pluripotent stem cells were analyzed for germ layer markers after 3–4 weeks of differentiation in medium consisting of Dulbecco's modified Eagle's medium/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. Scale bar = 75 μm. Abbreviations: miPS, mRNA-reprogrammed hiPSCs; riPS, retroviral-derived hiPSCs.

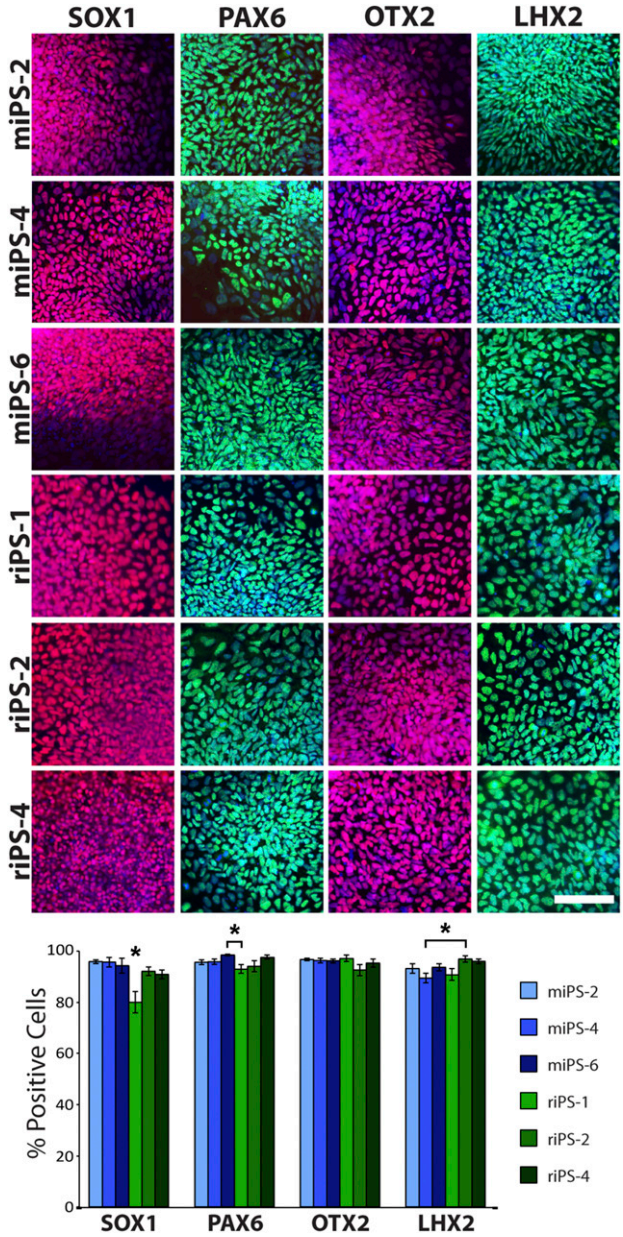

Figure 4. Establishment of a primitive neural fate from mRNA- and retroviral-reprogrammed cell lines. miPS and riPS cell lines expressed primitive neuroepithelial markers, including SOX1 and PAX6, at high efficiency within the first 10 days of differentiation. Furthermore, all human induced pluripotent stem cell 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 SOX1, PAX6, and LHX2 among some miPS and riPS lines; however, no correlation was observed between the mRNA and retroviral method of reprogramming. *, $p < .05$. Scale bar = 100 μm. Abbreviations: miPS, mRNA-reprogrammed hiPSCs; riPS, retroviral-derived hiPSCs.

with robust expression of retinal progenitor-associated markers such as CHX10 and PAX6 observed (Fig. 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. 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 after isolation of OV-like structures, at levels equal to or greater than their retroviral counterparts.

Differentiation of Specific Retinal Cell Types

Retinal progenitor cells of the optic vesicle have the ability to give rise to all the cell types of the retina in a precise and temporal

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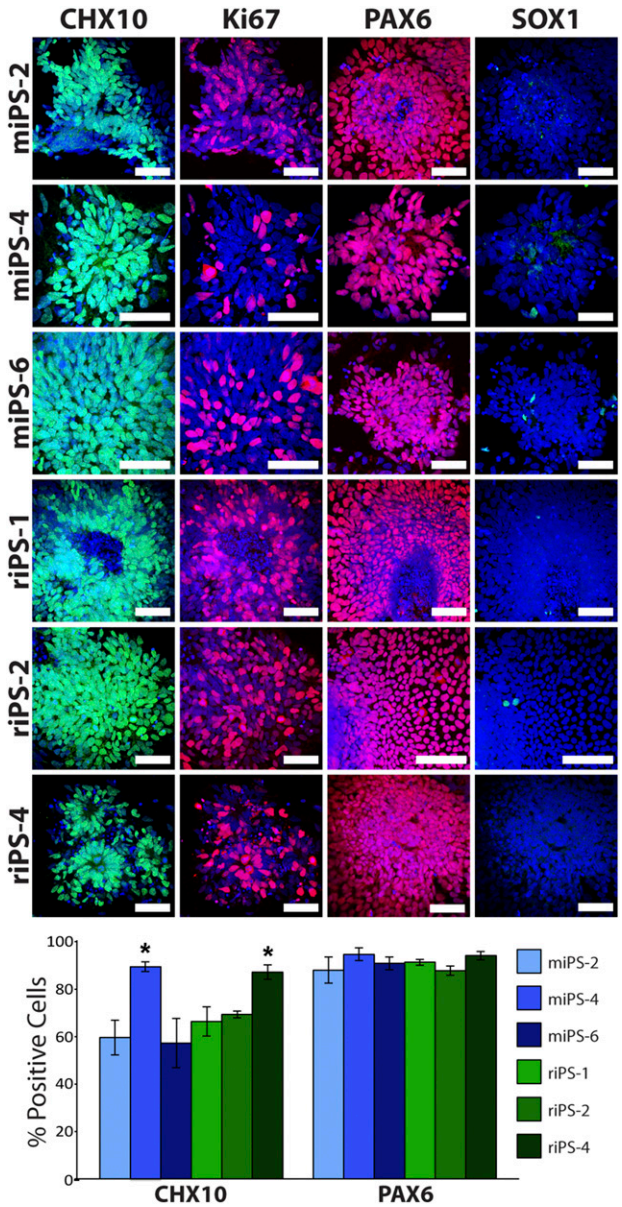

Figure 5. Differentiation of miPS and riPS cells to a retinal lineage. Following the establishment of an anterior neural fate, a subset of cells acquired a definitive retinal progenitor fate within 30 days of differentiation, as confirmed by the expression of retinal progenitor markers, including CHX10 and PAX6. The highly proliferative nature of these cells was demonstrated by the expression of Ki-67. Importantly, the 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 the human induced pluripotent stem cell lines, irrespective of the reprogramming method, and expression of PAX6 was consistent across all cell lines. *, $p < .05$. Scale bars = 50 μm. Abbreviations: miPS, mRNA-reprogrammed human induced pluripotent stem cells; riPS, retroviral-derived human induced pluripotent stem cells.

fashion [51–53]. Similarly, hiPSC-derived retinal progenitor cells have previously been demonstrated to give rise to all the major cell types of the retina in an orderly manner. 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

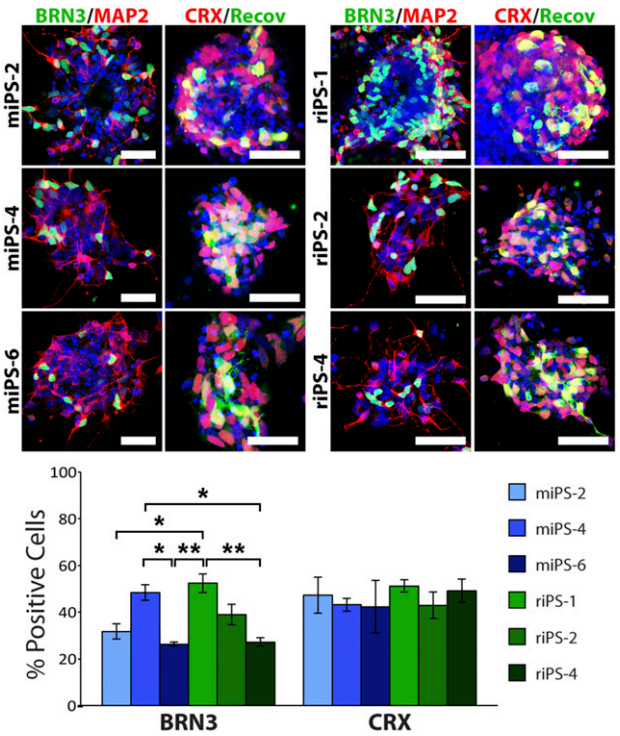

Figure 6. Generation of retinal neural cell types from human induced pluripotent stem cells. After approximately 70 days of differentiation, cells were analyzed for the expression of markers of retinal ganglion cells (BRN3/MAP2) or retinal photoreceptors (CRX/RECOVERIN). All cell lines were capable of generating these retinal cell types, although significant variability was observed in the expression of BRN3 among some miPS and riPS cell lines (*, $p < .05$; **, $p < .01$; ***, $p < .005$). Scale bars = 50 μm. Abbreviations: miPS, mRNA-reprogrammed human induced pluripotent stem cells; riPS, retroviral-derived human induced pluripotent stem cells.

associated with the onset of RPE differentiation was observed, as previously described [25, 28, 36, 54–64], across all cell lines, with no overt differences detected. These RPE cells became more abundant over the next 1–2 months and eventually gave rise to expandable monolayers of pigmented, hexagonal RPE-like cells from all the cell lines tested (supplemental online Fig. 1).

Alternatively, CHX10-positive OV-like structures were more likely to give rise to cells of the neural retina, particularly the more early born cell types, such as retinal ganglion cells and developing photoreceptor cells (Fig. 6), as previously documented [13, 14, 16, 20, 21]. Interestingly, all lines of miPS cells were able to generate CRX-positive photoreceptor-like cells at statistically similar efficiencies, which correlated with the ability of retroviral-derived cells to yield photoreceptor-like phenotypes. Additionally, all miPS and riPS cell lines expressed a variety of genes associated with retinal ganglion cells, photoreceptors, and interneurons of the retina, as demonstrated by RT-PCR (supplemental online Fig. 2). However, both miPS and riPS cell lines exhibited significant differences in their ability to give rise to BRN3-positive retinal ganglion cells, with some lines capable of yielding nearly one half (49.13% $\pm$ 2.95%) BRN3-positive retinal ganglion cells following enrichment for OV-like structures.

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.

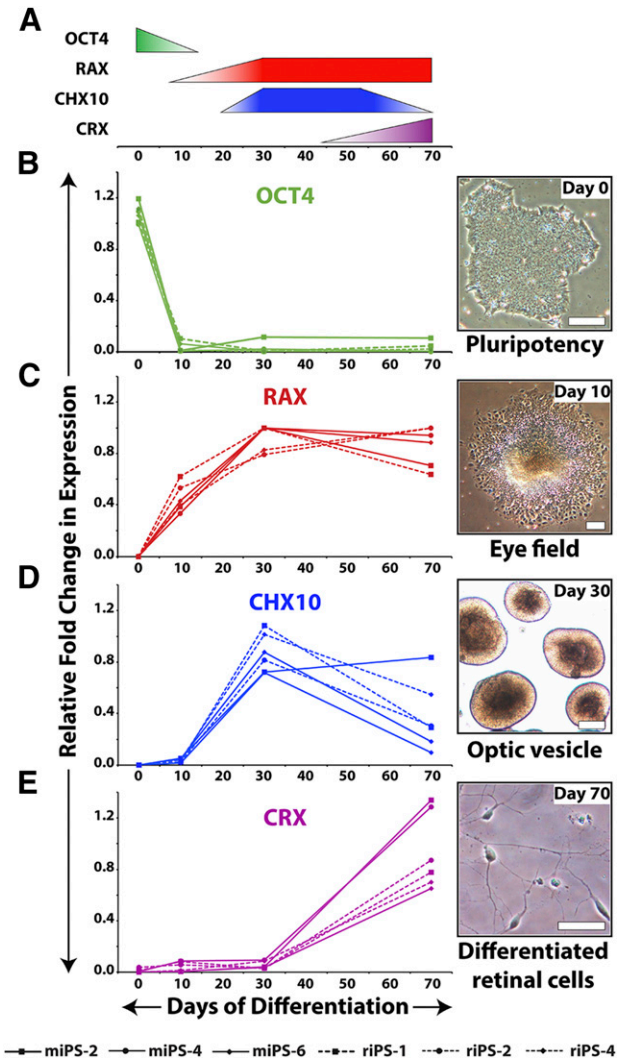

Figure 7. In vitro retinal differentiation of human induced pluripotent stem cells (hiPSCs) recapitulates known stages of retinogenesis. All lines of hiPSCs were assessed for expression of stage-specific genes during the course of retinal differentiation **(A)**. quantitative reverse transcription polymerase chain reaction analyses demonstrated that although the expression of pluripotency factor OCT4 decreased on differentiation, transcription factors RAX and CHX10 were expressed at slightly later time points 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, the results demonstrated that all miPS and riPS cell lines recapitulated stage-specific patterns of gene expression typically associated with human retinogenesis. Scale bars = 200 μm **(B–D)** and 50 μm **(E)**. Abbreviations: miPS, mRNA-reprogrammed human induced pluripotent stem cells; riPS, retroviral-derived human induced pluripotent stem cells.

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 that 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 (Fig. 7). 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 10 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.

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 [4, 12–14, 16, 21–26, 65, 66]. However, such efforts were often complicated by the 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 our study for the first time demonstrate the feasibility of deriving retinal cells from mRNA-reprogrammed hiPSCs and, as such, will likely facilitate future efforts toward the development of translational and therapeutic applications of hiPSC-derived retinal cells.

Traditionally, hiPSCs have been reprogrammed from a fibroblast source through the use of viral vectors encoding for pluripotency-associated transcription factors [7, 33, 35]. Although these approaches were straightforward and effective, the use of viral vectors was also associated with numerous undesirable features, in particular, 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. Although such constitutive expression might assist in the efficient reprogramming of fibroblasts to an hiPSC fate, it could also hinder efforts to direct the differentiation of resultant hiPSCs. Furthermore, viral delivery of reprogramming vectors has also been associated with the risk of insertional mutagenesis, because these vectors incorporate into the host genome in a random fashion [37–40]. The effects of these genomic insertions could interrupt certain genes necessary for differentiation to desired cellular lineages or even disrupt tumor suppressor genes, leading to uncontrolled growth of cells.

More recent efforts to generate hiPSCs have focused on methods to minimize the likelihood of such issues, while still maintaining or improving reprogramming efficiency. Perhaps most notably, the use of nonviral methods such as episomal vectors has become more widely used in recent years [34, 67]. Episomal vectors are ostensibly nonintegrating 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, because of 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 these concerns. The recent advent of mRNA-based reprogramming strategies suitably eliminates these concerns, as the DNA

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transgene-free source would prevent genomic integration, and the mRNA nature of these vectors would be rapidly degraded within the cell.

Although the importance and utility of mRNA reprogramming for the generation of hiPSCs has been well-documented [47, 48], the ability of these cells to faithfully recapitulate existing differentiation protocols and yield a full complement of retinal phenotypes has not been previously 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 and its highly organized nature [18, 27, 30, 32, 68]. Recent efforts have worked toward the clinical applications of human pluripotent stem cells, including hiPSCs, for the potential treatment of retinal degenerative disorders [58, 61, 69]. 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 the protocols for differentiation to the desired cellular lineages are comparably efficient, if not enhanced, when using mRNA-reprogrammed hiPSCs compared with their virally derived counterparts.

The results of the present study help to demonstrate that the nonintegrating, DNA-free nature of mRNA reprogramming is likely to be an important development for the generation and application of hiPSC-derived retinal cells. hiPSCs derived through mRNA reprogramming strategies were demonstrated to yield retinal cells at a similar efficiency compared with their virally derived counterparts, as significant differences between the cell line types 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, because genomic variations between different samples would likely introduce increased variability between the lines [13, 70, 71]. Thus, the present study explored the use of hiPSCs derived from both mRNA and retroviral reprogramming strategies using the identical source material of BJ fibroblasts, which have previously been used by several other groups for the derivation of hiPSCs [33, 35, 48, 72]. Thus, any potential differences among the cell lines could not be attributed to the origin of the cells before reprogramming. A certain degree of variability can also be expected, apart from the reprogramming method itself; thus, to account for this type of variability, three different lines of hiPSCs were generated through each reprogramming method and all six lines of hiPSCs were differentiated through each stage of retinal specification.

After a total of 30 days of differentiation, it has been previously demonstrated that hiPSC-derived OV-like structures might be readily identified and manually enriched, with these cells capable of giving rise to all major cell types of the retina [4, 12–16, 20, 21]. Similarly, hiPSCs derived through mRNA-reprogramming methods were capable of generating these OV-like structures within 30 days of differentiation. The retinal progenitor-associated transcription factor CHX10 was expressed in most cells in all lines of hiPSCs at this stage, although significant differences did exist between some lines. No correlation was observed in the expression of CHX10 between mRNA- and retroviral-derived lines of hiPSCs; however, 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 the other cell lines tested, consistent with previous studies with the H9 (WA09) human embryonic stem cell line [13, 20]. Those lines of hiPSCs with a lower percentages of cells expressing CHX10 are not likely to be less retinal in nature, because further differentiation of these cells yielded retinal neurons at similar efficiencies by a total of 70 days of differentiation. Rather, these variations might represent slight differences in the developmental timing of these cell lines during differentiation [20].

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, in particular, retinal ganglion cells (RGCs) and developing photoreceptor cells [51–53]. Interestingly, these are two of the earliest generated retinal cell types in vivo, suggesting that this differentiation paradigm is biased toward the generation of early born cell types. Although no significant differences were observed in the differentiation of photoreceptor-like cells across all cell lines tested, significant differences did exist in the generation of retinal ganglion cells, with some lines of hiPSCs generating nearly one half BRN3-positive RGCs and others as little as 20%. These differences could not be attributed to the reprogramming method itself and were 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 [73]. 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 ability to give rise to a desired cell type and selecting lines of hiPSCs carefully, because some lines might be more appropriate for certain applications than others.

Conclusion

The overall results of the present 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 nonintegrating, 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 will 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 because the differentiated retinal progeny derived from both human embryonic and induced pluripotent stem cells are becoming closer to clinical applications for disease modeling [10, 13, 22, 23, 25, 26], pharmacological screening [13, 22, 74], and cell replacement [18, 28, 30, 32, 75]. 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 [16, 62, 76, 77], and these approaches will need to be combined with appropriate reprogramming strategies, such as mRNA reprogramming.

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Author Contributions

A.S.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; S.K.O.: conception and design, collection and/or assembly of data; K.B.L.: collection and/or assembly of data; J.S.M.: conception and design, financial support, provision of study material or patients, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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