



Generation of Transplantable Retinal Photoreceptors from a Current Good Manufacturing Practice-Manufactured Human Induced Pluripotent Stem Cell Line

JIE ZHU,^a JOSEPH REYNOLDS,^a THELMA GARCIA,^a HELEN CIFUENTES,^a SHEREEN CHEW,^a
XIANMIN ZENG,^{a,b} DEEPAK ASHOK LAMBA ^a

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^aBuck Institute for Research on Aging, Novato, California, USA; ^bNxCell Inc, Novato, California, USA

Correspondence: Deepak A. Lamba, M.B.B.S., Ph.D., 8001 Redwood Blvd, Novato, California 94945, USA. Telephone: (415) 209 2076; e-mail: dlamba@buckinstitute.org

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ABSTRACT

Retinal degeneration often results in the loss of light-sensing photoreceptors, which leads to permanent vision loss. Generating transplantable retinal photoreceptors using human somatic cell-derived induced pluripotent stem cells (iPSCs) holds promise to treat a variety of retinal degenerative diseases by replacing the damaged or dysfunctional native photoreceptors with healthy and functional ones. Establishment of effective methods to produce retinal cells including photoreceptors in chemically defined conditions using current Good Manufacturing Practice (cGMP)-manufactured human iPSC lines is critical for advancing cell replacement therapy to the clinic. In this study, we used a human iPSC line (NCL-1) derived under cGMP-compliant conditions from CD34+ cord blood cells. The cells were differentiated into retinal cells using a small molecule-based retinal induction protocol. We show that retinal cells including photoreceptors, retinal pigmented epithelial cells and optic cup-like retinal organoids can be generated from the NCL-1 iPSC line. Additionally, we show that following subretinal transplantation into immunodeficient host mouse eyes, retinal cells successfully integrated into the photoreceptor layer and developed into mature photoreceptors. This study provides strong evidence that transplantable photoreceptors can be generated from a cGMP-manufactured human iPSC line for clinical applications. *STEM CELLS TRANSLATIONAL MEDICINE* 2018;7:210–219

SIGNIFICANCE STATEMENT

This study describes the generation of retinal cells including photoreceptors, as well as retinal pigmented epithelial cells from a current Good Manufacturing Practice compliant human induced pluripotent stem cell (iPSC) line after 12 weeks of directed differentiation using a novel small-molecule induction protocol. This study provides proof-of-concept that clinically compliant human iPSC lines can be used for making clinically relevant retinal cell types especially photoreceptors, thereby supporting the advancement of cell replacement therapy for preclinical studies and clinical applications.

INTRODUCTION

Various inherited and acquired retinal degenerative disorders often lead to the loss of photoreceptors (rods and cones) in the retina leading to severe and permanent visual loss. This is due to the fact that the adult mammalian retina lacks any significant regenerative potential. In the past few decades, significant progress has been made in the understanding of the pathogenesis of a range of retinal degenerative diseases. However, currently available treatment options are very limited and mainly palliative. Cell replacement therapy is a promising therapeutic strategy for retinal degenerative diseases especially following the

discovery of pluripotent stem cell sources. A number of research groups have shown the potential of using either human embryonic stem cells (hESCs) or induced pluripotent stem cell (iPSCs) toward generation of various retinal cells [1–6]. ESC or iPSC derived retinal photoreceptors were tested for their integration potential in either normal or degenerative retinas of murine models of retinal degeneration conditions and have shown various levels of survival, integration and visual function restoration [4, 7, 8]. Due to the ethical concerns associated with using hESCs in patients clinically, iPSCs generated from the somatic cells of the donors including the patients are considered a good alternative cellular source. iPSCs have

a number of advantages including the ability to generate banks from super donor pools which match large population groups [9], as well the eventual future use of patient's own cells to generate matched differentiated cells [10]. There are some drawbacks to iPSCs for cell therapy including the use of oncogenes in their generation as well as incorporation of mutations during the process of generation of these lines [11, 12] though some of these concerns also exist for hESC lines in culture [13]. Thus, clinically-compliant iPSCs may require thorough validation prior to clinical use. Recent advancements in pluripotent stem cell technology have shown the success of generating integration-free iPSC lines using either episomal plasmids, Sendai virus-based vectors or piggyBac transposons which are safer for clinical applications [14–19]

There is an urgent demand for a bank of iPSC lines produced under current Good Manufacturing Practice (cGMP) conditions to forward the stem cell research toward clinical translation. As of today, there are several cGMP-compatible iPSC lines reported and few of them are available for evaluation [20, 21]. In this study, we used a clinically compliant iPSC line (NCL1) to make retinal photoreceptors using our small molecule-based retinal induction protocol. NCL1 line was developed under cGMP-compliant condition including tissue sourcing, manufacturing, testing and storage. The line was generated from isolated cord blood-derived CD34+ cells which were reprogrammed using episomal plasmids in feeder-free culture condition [20]. The iPSCs meet the requirement of clinical application and can be used for differentiation into clinically relevant cells, holding potential for clinical applications.

In this study, we describe the generation of retinal cells including photoreceptors, as well as retinal pigmented epithelial (RPE) cells from NCL1 line after 12 weeks of directed differentiation using a novel small-molecule induction protocol. The protocol also allowed for generation of three-dimensional (3D) retinal organoids in low-attachment culture conditions. Upon subretinal transplantation of mono-layer cultured cells, the iPSC-derived retinal cells were able to integrate into immunodeficient host mouse retina at 8 weeks post transplantation and displayed mature photoreceptor morphology and expressed markers specific for mature functional photoreceptors. This study provides proof-of-concept that clinically compliant human iPSC lines can be used for making clinically relevant retinal cell types especially photoreceptors, thereby supporting the advancement of cell replacement therapy for preclinical studies and clinical applications.

MATERIALS AND METHODS

iPSC Culture and Retinal Induction

The NCL1 line was obtained from NxCell Inc (Novato, CA). The cells were maintained in Essential 8 basal medium (ThermoFischer Scientific, Waltham, MA) supplemented with 1% Essential 8 supplement (ThermoFischer Scientific, Waltham, MA) and 1% Penicillin Streptomycin Amphotericin B (Lonza, Walkersville, MD). Cells were grown on Matrigel-coated (BD Biosciences, San Jose, CA) culture plates in a 37°C incubator with 5% CO₂ and 5% O₂. Retinal differentiation was induced in iPSCs in medium that contains Dulbecco's modified Eagle medium (DMEM)/F-12 1:1 (GE Healthcare Life Sciences, Marlborough, MA) with 10% Knockout Serum Replacement (KSR, ThermoFischer Scientific), 1% Penicillin Streptomycin Amphotericin B, 1% Sodium Pyruvate (ThermoFischer Scientific), 1% Sodium Bicarbonate (ThermoFischer Scientific), 1% HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) Buffer

(ThermoFischer Scientific), 1% MEM (Minimum Essential Medium) Nonessential Amino Acids (ThermoFischer Scientific), and 1% N1 Media Supplement (Sigma-Aldrich, St. Louis, MO). Two retinal induction protocols were used to compare relative efficiency of retinal induction. One protocol contains 10 ng/ml of human recombinant protein Dickkopf-1 (DKK1) (R&D Systems, Minneapolis, MN, <https://www.rndsystems.com>), 10 ng/ml of human recombinant Noggin (R&D Systems), and 10 ng/ml of human recombinant Insulin-like growth factor 1 (IGF1) (R&D Systems) (referred as DIN) as reported previously [7, 22]. The new small molecule induction protocol contains 2 μM of IWR1 (Sigma-Aldrich), 10 μM of SB431542 (Stemgent, Lexington, MA, <https://www.stemgent.com>), 100 nM of LDN193189 (Stemgent), and 10 ng/ml of human recombinant IGF1 (R&D Systems) (referred as ISLI). The two protocols were used to treat undifferentiated iPSCs in parallel for 5 to 7 days with daily medium change. Cells were then dissociated and passaged onto 6-well Matrigel-coated plates at a passaging ratio of 1:3 in Neural Stem Cell (NSC) medium that was comprised of DMEM/F-12 1:1 (GE Healthcare Life Sciences), 0.5% Fetal Bovine Serum (FBS, Atlanta Biologicals, Atlanta Biologicals, Flowery Branch, GA, <https://www.atlanta-biologicals.com>), 1% Penicillin Streptomycin Amphotericin B, 1% Sodium Pyruvate, 1% Sodium Bicarbonate, 1% HEPES Buffer, 1% MEM Non-Essential Amino Acids, and 1% of N1 media supplement. The neuro-retinal regions were manually separated from co-existing RPE cells, dissociated and cultured in Matrigel-coated 6-well plates as monolayer. The neural cells were maintained in NSC media and serially passaged using Accutase (Global Cell Solutions, North Garden, VA) at 1:3 ratio upon confluency. Some neuro-retinal regions were also picked and cultured in low-attachment culture plates to allow formation of retinal organoids. The remaining RPE cells were cultured in RPE medium that contains Minimal Essential Medium with Earle's Balanced salt solution (MEM/EBSS) (GE Healthcare Life Sciences) with 1% FBS, 1% Penicillin Streptomycin Amphotericin B, 1% Glutamax (ThermoFischer Scientific), 0.25 mg/ml Taurine (Sigma-Aldrich), 10 μg/ml Hydrocortisone (Sigma-Aldrich), and 0.0065 μg/ml Triiodo-Thyronine (Sigma-Aldrich), and 1% N1 media supplement indefinitely till the time of analysis.

Culture of 3D Retinal Organoids

Two to four weeks of retinal induction rosette-like neuronal clusters started to form in culture. The clusters were manually isolated and cultured in ultra-low attachment 6-well plates (ThermoFischer Scientific) in NSC medium to allow self-organization and differentiation. NSC medium was changed 2 to 3 times per week till the time of analysis.

Animal Care

The IL2r $\gamma^{-/-}$ mice (B6.129S4-Il2rgtm1Wjl/J) were purchased from Jackson Laboratories. All mice used in this study were housed and bred at the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited vivarium of The Buck Institute for Research on Aging in a Specific Pathogen Free facility and housed in individually ventilated cages on a standard 12:12 light cycle. All procedures were approved by the Buck Institute Institutional Animal Care and Use Committee (IACUC) and performed in accordance with the guidelines and regulations set forth by the committee.

Transplantation of Human iPSC-Derived Retinal Cells

Human iPSC-derived retinal cells were treated with fresh NSC medium with 1 μ M Thiazovivin (Selleck Chemicals, Houston, TX) 1 hour prior to dissociation. The cells were then dissociated with Accutase. The final cell suspension was prepared in NSC medium containing \sim 500,000 cells per microliter. The cell suspension was kept on ice until transplantation.

Transplantation procedure is based on previously described technique [23]. Animals were anesthetized for 15 minutes with 2% Isoflurane inhalation. Pupils were dilated using a mixture of 5% Phenylephrine (Arcos Organics, Geel, Belgium) and 1% Tropicamide (Alfa Aesar, Ward Hill, MA) and anesthetized with topical 0.5% Proparacaine hydrochloride ophthalmic solution (Akorn, Vernon Hills, IL). Following testing the depth of anesthesia, a small hole was made on the peripheral cornea using a 30G needle visualized using a dissecting microscope (Olympus, San Jose, CA). A pulled graduated glass pipet (Wiretrol II, 5-0000-2005, Drummond Scientific Company, Broomall, PA) containing 1–1.5 μ l of cell suspension (500,000–750,000 cells) was inserted into the anterior chamber of the eye through the hole, advanced gently across the iris until it reached the subretinal space of the eye. The cells were then slowly injected into the subretinal space. The pipet was withdrawn slowly to minimize backflow of cell suspension. The Neomycin and Polymyxin B sulfates and Bacitracin Zinc Ophthalmic Ointment (Akorn) was applied to the eye and mice were monitored for full recovery and general health until euthanasia.

Immunocytochemistry and Immunohistochemistry

Retinal cells, RPE cells, retinal organoids, and transplanted eyes were collected and fixed with 4% Paraformaldehyde for 30 minutes to 2 hours at room temperature followed by thorough wash in 1X Phosphate-Buffered Saline (PBS) buffer. The retinal organoids and eye samples were embedded with embedding medium that contains 7.5% gelatin (Sigma) and 15% sucrose (Sigma) in 1X PBS buffer. Cryo-embedded eye samples were sectioned to 14 μ m thickness. Fixed cells, retinal organoids, and eye sections were analyzed with the antibodies listed in Supporting Information Table S1. Secondary antibody staining was done using the corresponding Alexa 488, 555, 594, 647 fluorescent-tagged antibodies (Invitrogen, Molecular Probes, ThermoFischer Scientific). 0.1% DAPI (Enzo Life Sciences, Farmingdale, NY) was used to stain the nuclei and samples were mounted using Fluoromount-G medium (Electron Microscopy Science, Hatfield, PA) for analysis. Images were taken with Zeiss LSM510 confocal microscope.

Real Time PCR

Total RNA was extracted from the cells using the Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA). All steps were done according to the manufacturer's recommended instructions. cDNA was reversed transcribed using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) as per the manufacturer's instructions in a T100 Thermal Cycler (Bio-Rad). The qRT-PCR was performed in CFX Connect system (BioRad) for various genes (See Supporting Information Table S2 for primer sequences). Results were normalized to β -actin levels. PCR gel analysis was done by running PCR products on 1.5% agarose gels (BioExpress, Kaysville, UT) to determine the presence or absence of PCR products.

RESULTS

Small Molecule-Based Differentiation Protocol Promotes Eye-Field Induction

Our previous work has shown that retinal differentiation can be successfully induced in hESCs and iPSCs via inhibition of Wnt and BMP signaling [3]. Human recombinant proteins DKK1 (Wnt signaling inhibitor) and Noggin (BMP signaling inhibitor), together with human recombinant IGF1 were used to induce retinal differentiation in our previous protocol, referred as DIN in this study. In order to establish a more defined and cost-effective protocol for retinal cell production, we replaced the DKK1 and Noggin with small molecule inhibitors directed against these pathways and tested the efficiency of retinal induction between the two protocols. We used a combination of small molecule inhibitors IWR1 (Wnt signaling inhibitor), SB431542 (TGF β signaling inhibitor), LDN193189 (BMP signaling inhibitor), in addition to human recombinant protein IGF1. The new retinal induction protocol is referred as ISLI in this study (Fig. 1A). The NCL1 human iPSC line was maintained in chemically defined Essential 8 medium (E8) (Fig. 1C). Cells were plated for 5–7 days prior to retinal induction. Cells were treated with either DIN or ISLI containing media for 5 days with daily medium change (Fig. 1A). At 5 days of retinal induction, a significant decrease in expression of the key pluripotency marker *OCT4* was observed in ISLI but not in DIN treated cells (Fig. 1B). On the other hand, we observed similar increases in expression of eye-field transcription factors *LHX2*, *PAX6* and *RX* under both (DIN and ISLI) culture conditions by qRT-PCR (Fig. 1B). After 5 days of retinal induction, cells were split into Matrigel coated 6-well plates and cultured in NSC medium for the rest of the culture period. At 14 days of retinal induction, qRT-PCR analysis showed a further decrease in expression of *OCT4* and similar expression of eye-field transcription factors *LHX2*, *PAX6*, and *RX* in both ISLI and DIN treated cells (Fig. 1B). An elevated expression of RPE-specific transcription factor *MITF* was also detected in differentiating cells treated with either DIN or ISLI at this stage, indicating the differentiation of RPE cells in culture (Fig. 1B). The above data shows that the small molecule-based protocol is as efficient as the recombinant protein protocol in eye-field induction of human pluripotent stem cells.

Neuro-Retinal and RPE Differentiation of Small Molecule-Treated iPSCs

Rosette-like retinal clusters formed in cultures at 3 weeks of differentiation (Fig. 1D). Those neural clusters were manually picked up and expanded. By 6 weeks of directed differentiation, optic-cup like retinal organoids was clearly seen in retinal cell culture (Fig. 1E). The neural cells were cultured in NSC medium for extend time period. Upon immunocytochemical (ICC) analysis at 6 weeks of retinal induction, we observed that the cells expressed retinal stem/progenitor cell marker, *LHX2* (87% \pm 1% of total DAPI stained cells), and retinal stem cell, ganglion cell, and amacrine cell marker, *PAX6* (71% \pm 4% of total DAPI stained cells) (Fig. 2A, 2B). A subset of cells also expressed *BRN3*, a marker of differentiated retinal ganglion cells (11% \pm 1% of total DAPI stained cells) (Fig. 2C). At this stage, photoreceptor differentiation was often observed in localized clusters. Within those photoreceptor-enriched areas, 87% \pm 2% cells expressed pan photoreceptor marker *OTX2* (Fig. 2D), 82% \pm 4% expressed *CRX* (Fig. 2E), and 19% \pm 1% of total DAPI stained cells expressed *RECOVERIN* (Fig. 2F). Upon further differentiation for 12 weeks,

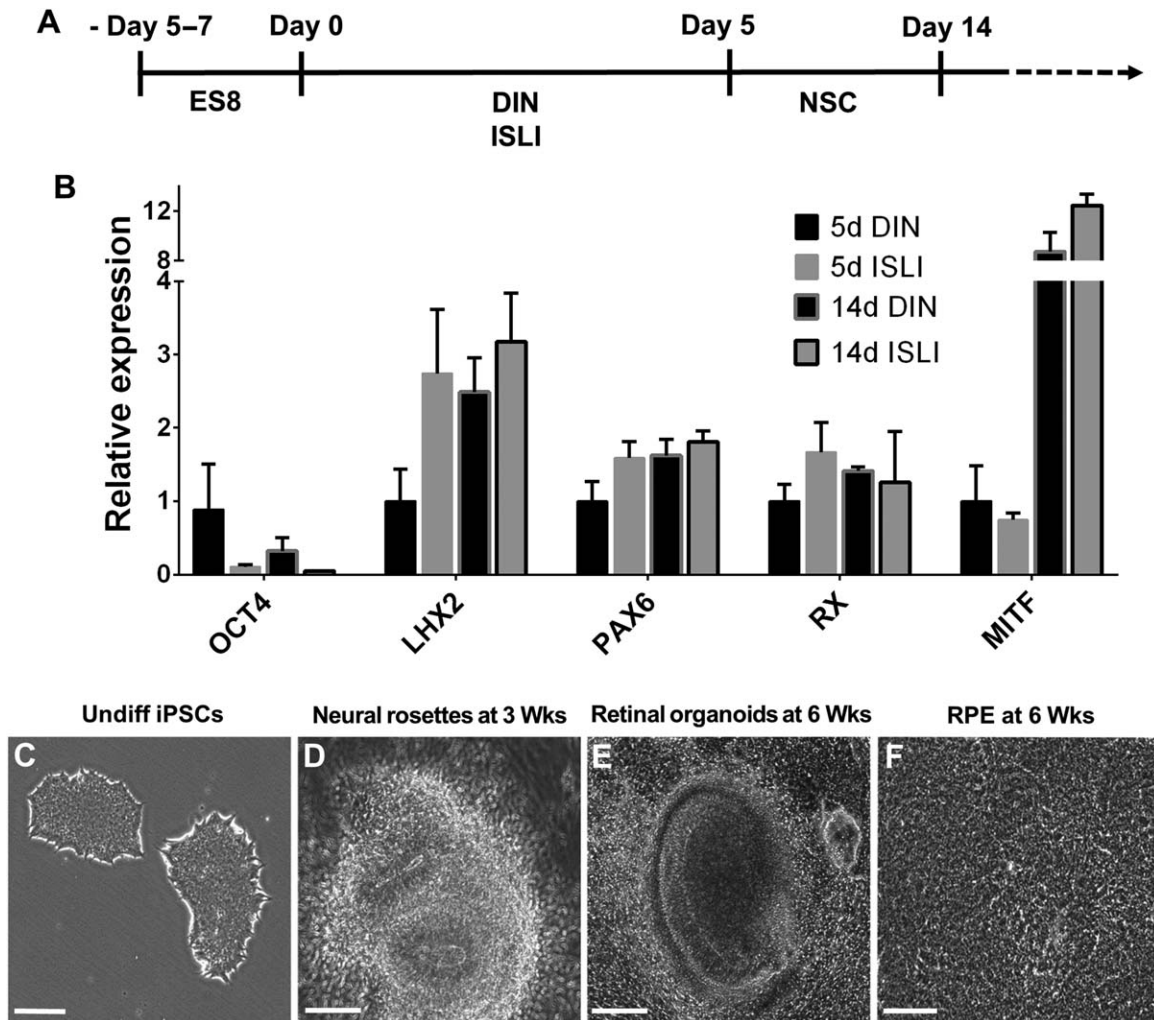
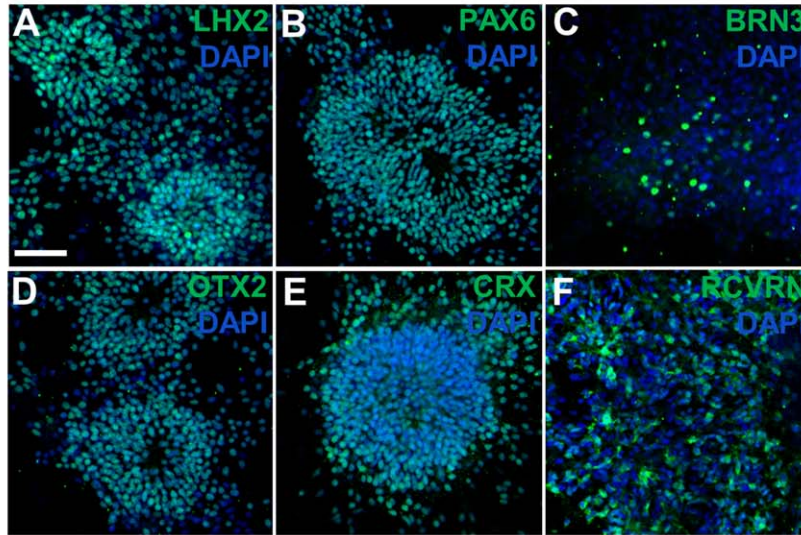


Figure 1. Small molecule-based differentiation protocol promotes eye-field induction. **(A):** Schematic diagram showing the timeline of retinal differentiation of human pluripotent cells. DIN represents the human recombinant protein-based protocol; ISLI represented the small-molecule based differentiation protocol. **(B):** Quantitative Real-time PCR data comparing gene expression relative to 5-day DIN treatment showing that the ISLI differentiation protocol worked as efficiently as the previously reported DIN protocol. Downregulation in expression of pluripotency marker *OCT4* and upregulation in expression of early eye-field transcription factors *LHX2*, *PAX6*, and *RX* genes were induced in differentiating human iPSCs at 5 and 14 days of directed differentiation. Upregulation in expression of *MITF*, a RPE specific gene, was detected at 14 days of directed differentiation with both protocols. **(C–F):** Representative brightfield images of current Good Manufacturing Practice-generated undifferentiated human iPSCs (C), neural rosettes in monolayer culture at 3 weeks of differentiation (D), retinal organoid-like cluster in monolayer culture at 6 weeks of differentiation (E), and RPE monolayer culture at 6-weeks (F). Scale bars = 100 μ m. Abbreviations: iPSCs, induced pluripotent stem cells; NSC, neural stem cell; RPE retinal pigment epithelium.

approximately half the cells in the plate expressed pan markers of photoreceptors OTX2 (52% \pm 6% of total DAPI stained cells in the plate) (Fig. 2G) and RECOVERIN (43% \pm 3% of total DAPI stained cells) (Fig. 2H). Cells in culture were also positive for AIPL1, a marker for mature photoreceptors (50% \pm 5% of total DAPI stained cells) (Fig. 2I). Of these, a subpopulation of cells expressed rod specific markers, NRL (14% \pm 2%) (Fig. 2J) and cone specific markers THRB (TR β 2, 17% \pm 2%) (Fig. 2K) and Cone Arrestin (5% \pm 1%) (Fig. 2L), indicating the ongoing rod and cone specification in culture at 12 weeks of directed differentiation. PCR analysis further confirmed the expression of retinal stem/progenitor/inner neuronal cell markers, *PAX6*, *ASCL1*, and *LHX2*, as well as a set of genes expressed in developing and differentiated photoreceptors including *BLIMP1*, *RCVRN*, *NRL*, *NEUROD1*, *THRB*(TR β 2), and *RXRG*, in iPSC-derived retinal cells at 12 weeks of differentiation (Fig. 2M).

Purified RPE cell cultures were also established separately by manual selection (Fig. 1F). These RPE cells were further cultured for 8 weeks to promote differentiation and maturation using methods previously described [24]. At the end of eight weeks, the cells displayed typical cobblestone morphology and pigmentation (Fig. 3A). The cells were further analyzed by PCR for various RPE cell-specific markers. The cells expressed various immature and mature RPE genes including *MITF*, *BEST1*, *PMEL17*, *RPE65*, *TYR*, *ALDH1A3*, *BMP4*, *PRNP*, *RDH5*, *SIL1*, *SLC4A2*, and *TIMP3* (Fig. 3B). Upon staining, the cultured cells expressed the RPE-specific transcription factor MITF along with OTX2 (Fig. 3C–3F). The cells were also stained for two mature RPE-specific markers RPE65 and Bestrophin (Fig. 3G–3J). The above data confirm that the small molecule based-retinal differentiation protocol leads to generation of various neuro-retinal and RPE cells in mono-layer cultures from cGMP-compliant iPSCs.

6 Wks of differentiation



12 Wks of differentiation

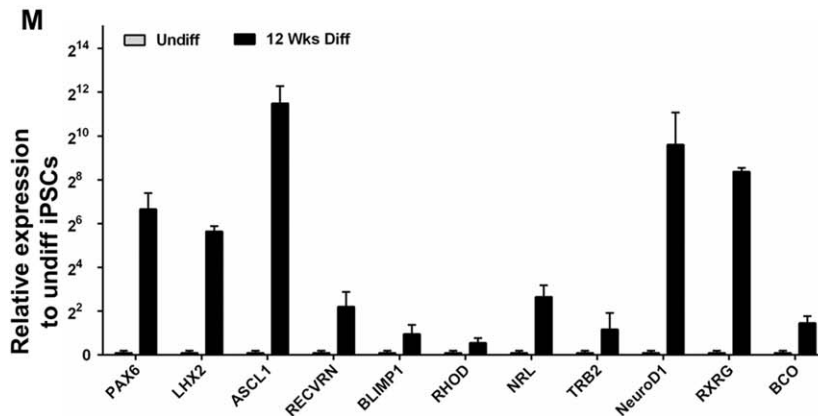
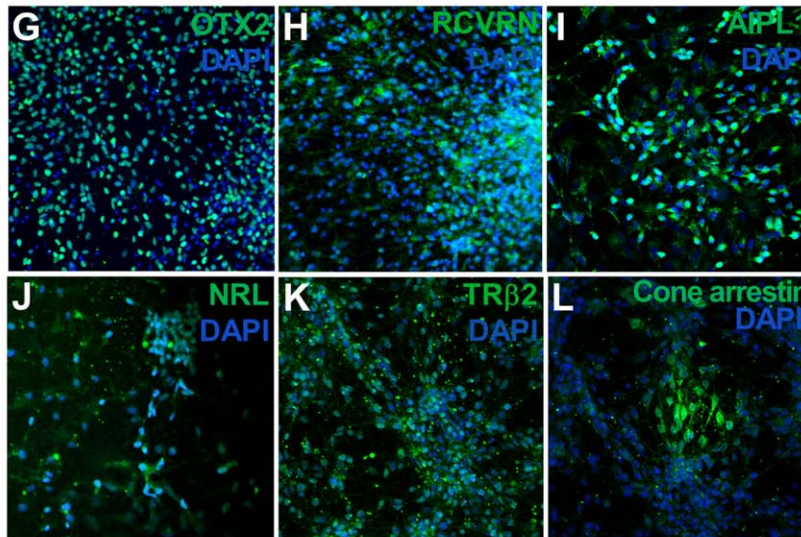


Figure 2. Neuro-retinal differentiation of small molecule-treated iPSCs. **(A–F):** Immunocytochemical analysis of retinal differentiation of human iPSCs in monolayer culture at 6 weeks of small molecule-induced differentiation. The majority of cells (70%–80%) in culture expressed retinal stem/progenitor marker, LHX2 (A), and retinal stem cell, ganglion cell and amacrine cell marker, PAX6 (71% ± 4% of total DAPI stained cells) (B) at this differentiation stage. In addition, cells expressed markers of retinal ganglion cells, BRN3 (C), pan-photoreceptor markers OTX2 (D), CRX (E), and RECOVERIN (F). **(G–L):** At 12 weeks of differentiation, cells in the plate were stained for pan-photoreceptor markers, OTX2 (G) and RECOVERIN (H) along with other immature photoreceptor marker, AIPL1 (I). Additionally, cells expressed both rod photoreceptor specific marker NRL (J) and cone photoreceptor specific marker TRβ2 (K) and cone arrestin (L). **(M):** Quantitative Real-time PCR data showing the expression of retinal stem cell, ganglion cell, and amacrine cell marker, PAX6, retinal stem cell markers, LHX2 and ASCL1 and a set of genes expressed in developing and differentiated photoreceptors, BLIMP1, RCVRN, TRB2 (THRB), RHOD, NRL, NEUROD1, BCO, and RXRγ at 12 weeks of retinal induction. Scale bars = 50 μm in (A–L). Abbreviation: iPSCs, induced pluripotent stem cells.

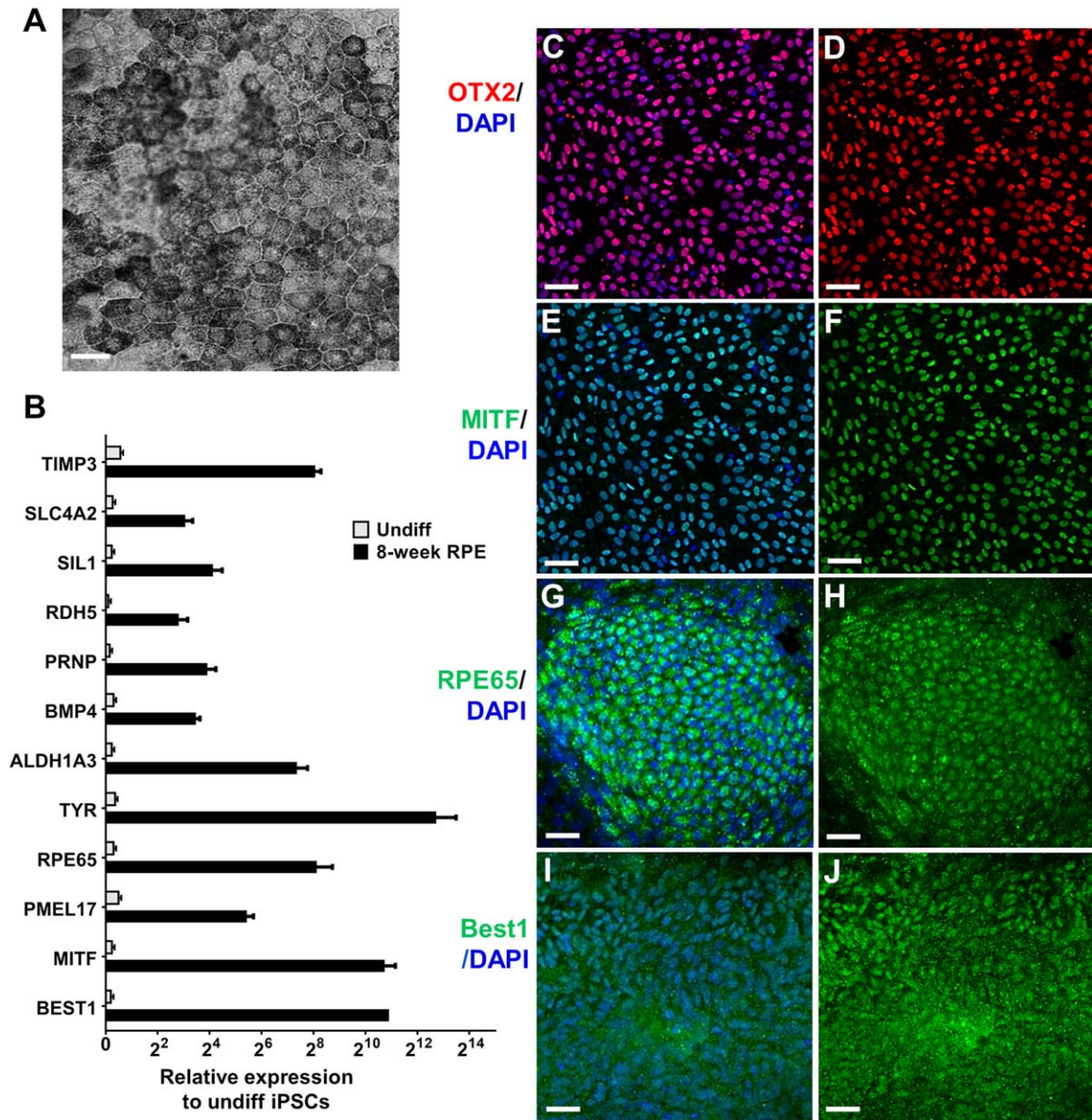


Figure 3. RPE differentiation of small molecule-treated iPSCs. (A): Representative brightfield microscopy image showing RPE cultures exhibiting typical cobblestone morphology and pigmentation at 8 weeks of differentiation. (B): Quantitative Real-time PCR data showing the expression of various immature and mature RPE genes including *BEST1*, *PMEL17*, *RPE65*, *MITF*, *TYR*, *ALDH1A3*, *BMP4*, *PRNP*, *RDH5*, *SIL1*, *SLC4A2*, and *TIMP3* in cells. (C–F): Immunocytochemical analysis showing most of the cells in culture expressed RPE-specific transcription factors OTX2 (C, D) and MITF (E, F). (G–J): RPE-specific markers RPE65 (G, H) and Bestrophin (I, J) were also expressed in cells at 8 weeks of culture. DAPI (blue) stains the nuclei of the cells. Scale bars = 20 μ m. Abbreviations: iPSCs, induced pluripotent stem cells; RPE retinal pigment epithelium.

3D Retinal Organoids Derived from iPSCs Following Low-Attachment Culture

At 4 weeks following retinal induction with ISLI, rosette-like retinal clusters were manually picked and cultured in low-attachment conditions in NSC medium to allow the formation of self-organized retinal organoids from the iPSC-derived retinal cells (Fig. 4A). IHC analysis targeted against markers of retinal and photoreceptor precursor cells was carried out at 6-weeks stage. The retinal organoids formed laminated structure similar to the retina in vivo. The organoids were predominantly comprised of cells expressing PAX6 (Fig. 4B). There was a polarized distribution of CRX⁺ and OTX2⁺ cells in the outer layer of the retinal organoids indicating the differentiation and lamination of photoreceptors at this stage (Fig. 4C, 4E).

Differentiated retinal ganglion cells (BRN3⁺ and ISLET1⁺) and other inner retinal cell types (amacrine cells, bipolar cells and horizontal cells) (ISLET⁺) were present in the inner side of the retinal organoids (Fig. 4D, 4E). Upon analysis at 12 weeks of differentiation, retinal organoids showed clear signs of maturation. A marked decrease in the number of PAX6⁺ cells was seen within the organoids (Fig. 4F) and majority of the cells in the outer layer of the organoids expressed several pan photoreceptor markers, OTX2, CRX, BLIMP1, RECOVERIN, and AIPL1 as compared with 6 weeks of differentiation (Fig. 4G–4K). The rod photoreceptor-specific marker NRL (Fig. 4L) and cone photoreceptor specific markers RXR γ (Fig. 4M) were also detected in the photoreceptor layer of the retinal organoid. The laminated

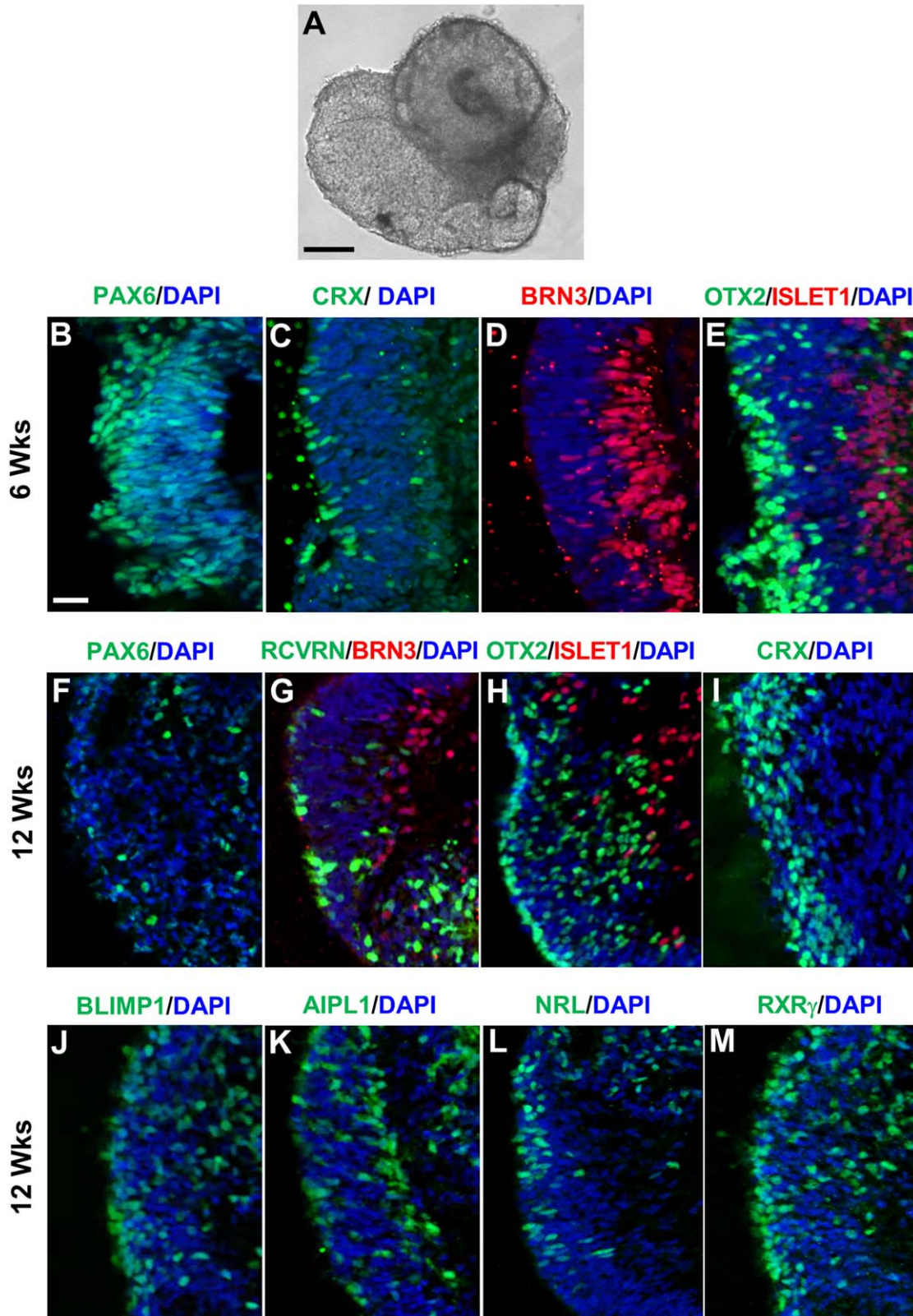


Figure 4. Three-dimensional Retinal organoids derived from induced pluripotent stem cells (iPSCs) via low-attachment culture. **(A):** Representative brightfield microscopy image showing optic cup-like organoids derived from human iPSC in low-attachment culture after 6 weeks of small molecule-based retinal induction. **(B–E):** Immunohistochemistry (IHC) analysis showing laminated retinal organoids at 6 weeks where-in the majority of cells express PAX6, a retinal stem/progenitor cell and inner retinal neuronal marker (B). CRX (C) and OTX2 (E) expressing photoreceptors show a polarized distribution on the apical side of the retinal organoids. BRN3 expressing retinal ganglion cells (D) and ISLET1 positive inner retinal cell types (E) were located in the inner side of the retinal organoids. **(F–M):** IHC analysis at 12 weeks of differentiation showing maintenance of retinal lamination with fewer PAX6 positive cells (F). Cells expressing photoreceptor markers, RCVRN (G), OTX2 (H), CRX (I), BLIMP1 (J), and AIPL1 (K) showed polarized distribution in the apical side of the retinal organoids. Cells in the photoreceptor layer show both rod photoreceptor specific marker NRL (L) and cone photoreceptor specific marker RXR γ (M). DAPI (blue) stains the nuclei of the cells. Scale bar = 200 μ m in (A); Scale bars = 50 μ m in (B–M).

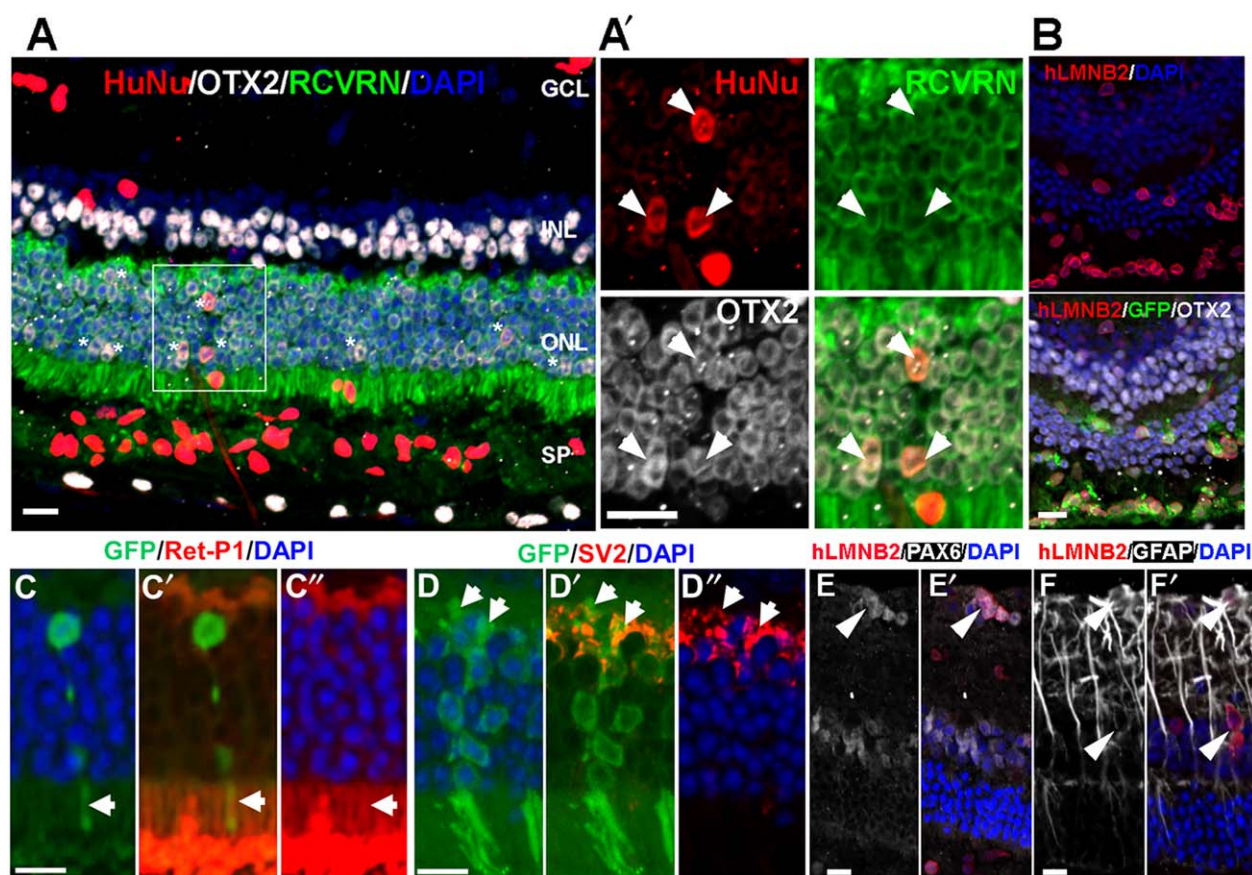


Figure 5. Integration of human induced pluripotent stem cells (iPSC)-derived retinal cells in $IL2r\gamma^{-/-}$ host retina at 8 weeks following transplantation. **(A-A')**: Immunohistochemistry analysis showing HuNu+ positive human iPSC-derived retinal cells (red and highlighted with *) integrated into the photoreceptor layer (ONL) of host $IL2r\gamma^{-/-}$ retina at 8 weeks following transplantation. Integrated HuNu+ human retinal cells in host ONL express pan-photoreceptor markers, OTX2 (white) and RCVRN (green, white arrows). **(B)**: Integrated GFP+ cells also stained for human nuclear membrane marker LMNB2 confirming the human origin of those cells. **(C-C')**: Representative image showing integrated cell with outer segments projecting toward the RPE layer co-expressing rhodopsin (white arrows). **(D-D')**: Representative image showing end-feet of integrated human photoreceptors expressing synaptic marker, SV2 (red, white arrows) in the outer plexiform layer of host retina. **(E-E', F-F')**: Representative images showing migrated PAX6+ inner retinal neurons co-expressing human LMNB2 marker as well as human LMNB2 co-expressing GFAP+ cells in the inner retina (marked by arrowheads). DAPI (blue) stains the nuclei of the cells. Abbreviations: GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; SP, subretinal space; V, vitreous. Scale bars = 10 μ m for (A-F').

retinal cups also showed clearly defined inner retinal layers comprised of BRN3+ and ISLET+ retinal ganglion cells and amacrine cells (Fig. 4G, 4H). The IHC data above on the time course of retinal differentiation and maturation within the organoids is consistent with the time-line of retinal differentiation in 3D retinal organoid culture previously reported by various groups [6, 25, 26]. It also closely recapitulates retinogenesis during human embryonic development [27]. Collectively, these data demonstrate that we are able to generate 3D retinal organoids that resemble developing human retina from cGMP-compliant iPSCs in culture.

Transplantation of iPSC-Derived Retinal Cells into Host Mice

Finally, we tested if the iPSC-derived retinal cells generated from NCL1 line have the ability to integrate into a host retina. Sixty-five to seventy-day differentiated retinal cells containing young photoreceptors were transplanted into the host retina. We and others have shown that young immature photoreceptors integrate maximally into a host retina [28–30]. To track the cells in host eye following transplantation, the retinal cells were infected with a replication-incompetent lentivirus expressing eGFP under the

hEF1 α ubiquitous promoter which drives expression of GFP by 50%–70% of all cells in the plate as previously described [7]. One day following infection, media was changed and cells cultured for an additional 5–7 days prior to transplantation into the subretinal space of 4–6 weeks old $IL2r\gamma^{-/-}$ mice via trans-corneal subretinal injection approach. $IL2r\gamma^{-/-}$ mice lack natural killer cells and have reduced number of mature lymphocytes [31]. These mice thus serve as a good humanized model for testing integration efficiency. Approximately 500,000 cells in 1 μ l volume were transplanted into the left eye of each recipient mouse which resulted in GFP+ donor cells covering approximately one-third of the subretinal space of host eye. We chose to analyze the cell survival and integration at 8 weeks post transplantation to allow time for maximal integration of the injected cells in the subretinal space of $IL2r\gamma^{-/-}$ host eyes [29]. At this time point, we observed human retinal cells integrated into the outer nuclear layer of the host retina (Fig. 5A–5A'). Recent reports suggest the potential transfer of GFP protein from the transplanted mouse photoreceptor cells to host mouse photoreceptors [32–34]. In order to rule out transfer of GFP from human stem cell-derived photoreceptors, we stained the transplanted sections with human-specific nuclear antigen (HuNu) as well as human specific antibody for Lamin B2 nuclear

membrane protein (hLMNB2). Individual integrated cells and cells present in the subretinal space of host eye were stained for HuNu (Fig. 5A, 5A') and hLMNB2 (Fig. 5B) confirming the human origin of those cells. We found 0.15% (SEM \pm 0.05%, $n = 3$) of transplanted cell integrated in the host retina based on human-specific markers. The integrated cells exhibited typical photoreceptor morphology with properly orientated photoreceptor segments and had their synaptic end-feet in the outer plexiform layer (Fig. 5C–5D''). The GFP+ cells expressed pan-photoreceptor markers, OTX2 (Fig. 5A, 5B) and Recoverin (Fig. 5A, 5A'). In addition, cells also expressed rod photo-transduction protein, Rhodopsin (Fig. 5C–5C'). We also looked for and detected the expression of synaptic marker, SV2, in the end-feet of GFP+ cells in the outer plexiform layer (Fig. 5D–5D'). We occasionally observed PAX6+ (Fig. 5E–5E') and GFAP+ (Fig. 5F–5F') human cells in the inner retina suggestive of Müller glia or astrocyte and ganglion or amacrine cell migration into host eye. We did not observe any evidence of tumors formation in the host eye (data not shown). This data shows that the iPSC-derived retinal cells are able to integrate into host retina and undergo maturation at 8 weeks following transplantation.

DISCUSSION

Stem cell-based therapies for retinal disease are currently transitioning from preclinical research to phase I/II clinical trials after decades of studies. A bank of thoroughly validated cGMP-compliant human iPSC lines is required for moving these therapies to the clinic. Here, we show that a clinically-compliant human iPSC line NCL1, made by a standardized cGMP-compliant protocol, can be efficiently directed to generate neuro-retinal cells including photoreceptors and RPE cells *in vitro*. Additionally, we described a refined small molecule-based retinal induction protocol via inhibition of Wnt, BMP, and TGF β signaling which minimizes variability we have previously seen using different lots, manufacturers, and batches of recombinant proteins. The time course of retinal differentiation in these human iPSCs with our protocol closely match what has been reported in previous studies [1, 3, 6, 7, 35]. The small molecule-based retinal induction protocol used in the study is more defined and cost effective. Though the components used in the current protocol to generate retina were not cGMP-grade, cGMP-validated equivalents are commercially available for most of them. We are currently looking to outsource the manufacture the small molecules used in the protocol to generate a cGMP-validated retinal induction media. We are also testing alternative ECM matrices as Matrigel is xeno-sourced and efforts are ongoing to optimize the use of alternatives including human fibronectin and vitronectin as these would work best in cGMP setting. One of the critical final steps of any cell differentiation methodology for *in vivo* application is to test the ability of these cells to integrate into host tissue. We confirmed that the iPSC-derived retinal cells generated using this modified small molecule (ISL1) protocol behaved similar to our original recombinant protein protocol in terms of their ability to integrate into a host retina. Transplanted photoreceptors were able to integrate into host and undergo maturation. The cells expressed rhodopsin in the outer segment region and synaptic protein SV2 at their end-feet. This data complements well with our recently published work showing long-term functional integration of hESC-derived retinal photoreceptors in Crx mutant mice over 9-months post-transplantation with visual improvements [29].

There has been a significant interest in generating and testing the applicability of 3D retinal organoids for both transplantation purposes [36] as well as disease modeling and retinal developmental studies [37, 38]. We tested if the small molecule based protocol could also generate such organoids. Self-organized laminated optic

cup-like retinal organoids could be efficiently generated by manual isolation from the cGMP-compliant iPSCs via low-attachment culture. The retinal organoids recapitulated the key structural features of developing retina, with photoreceptors (cones and rods) located in the outermost layer and inner retinal cell types located in the inner layer of retinal organoids, which is consistent with previously published studies [6, 21, 25, 26]. The organoids followed a maturation timeline as well with BRN3+ /ISLET1+ ganglion cells appearing at around 6–8 weeks and more mature photoreceptor markers such as RECOVERIN and AIPL1 appearing close to 12 weeks of floating culture. These iPSC-derived retinal organoids provide a great *in vitro* biological model system for disease modeling as well as developmental biology studies.

Photoreceptor replacement therapy will be critical for patients who have lost most of their light sensing cells. However, recent papers using primary young mouse photoreceptors transplanted into wild-type hosts have found that majority of GFP+ cells in the hosts were a result of cytoplasmic protein transfer with minimal nuclear migration [32–34]. Pearson et al did not see evidence of nuclear fusion, Singh et al did not find evidence of nuclear migration based on FISH analysis of X- and Y-chromosomes while Santos-Ferreira et al did not find any EDU labeled nuclei in the host retina following transplantation of labeled cells, though they did see rare Y-chromosome+ integrated cells by FISH. In our report, we observe cells labeled with two different human-specific nuclear markers, HuNu and human-specific LMNB2, in all layers of the host retina suggesting that human-stem cell derived retinal cells have the true capability to integrate into a host retina. Since these proteins are part of the nuclear matrix and envelope and previous studies shows that the transfer is not due to cell fusion, it is unlikely that these proteins transferred via cytoplasmic or exosome exchange between host and donor cells. This is consistent with our previous reports where we reported that integrated hESC-derived photoreceptors in Crx mutant retinas co-label with human-specific NRL antibody and HuNu [8]. Our report, however, does not completely exclude cytoplasmic transfer as mechanism for some of the GFP+ cells observed in the host retina. Further studies to better understand the mechanism for protein transfer and true integration capacity of cells will be critical prior to their clinical use.

CONCLUSION

Our work validates a directed differentiation strategy using small molecules to generate retinal cells from cGMP compliant human iPSCs. These cells in turn have the ability to migrate and integrate into a host mammalian retina and thus the potential to be used for cell replacement therapies.

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AUTHOR CONTRIBUTIONS

J.Z.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; J.R., T.G., H.C., and S.C.: collection and/or assembly of data, data analysis and interpretation; X.Z.: provision of study material; D.A.L.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, financial support, final approval of manuscript. NxCell, Inc provided the NCL-1 line free of cost for the studies in the paper. NxCell, Inc did not provide any funding nor did

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

X.Z. is affiliated to NxCell Inc and is partially compensated through it. All other authors indicated no potential conflicts of interest.

NOTE ADDED IN PROOF

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