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Optic Vesicle-like Structures Derived from Human Pluripotent Stem Cells Facilitate a Customized Approach to Retinal Disease Treatment

Jason S. Meyer^{1,*}, Sara E. Howden^{2,3,7}, Kyle A. Wallace¹, Amelia D. Verhoeven¹, Lynda S. Wright¹, Elizabeth E. Capowski¹, Isabel Pinilla⁹, Jessica M. Martin¹, Shulan Tian⁷, Ron Stewart⁷, Bikash Pattnaik^{4,5,6}, James Thomson^{2,3,7,8}, and David M. Gamm^{1,4,6}

¹Waisman Center, University of Wisconsin, Madison WI 53705

²Department of Cell & Regenerative Biology, University of Wisconsin, Madison WI 53705

³The Genome Center of Wisconsin, University of Wisconsin, Madison WI 53705

⁴Department of Ophthalmology and Visual Sciences, University of Wisconsin, Madison WI 53705

⁵Department of Pediatrics, University of Wisconsin, Madison WI 53705

⁶Eye Research Institute, University of Wisconsin, Madison WI 53705

⁷Morgridge Institute for Research, Madison WI 53706

⁸Department of Molecular, Cellular, & Developmental Biology, University of California Santa Barbara, Santa Barbara CA 93106

⁹Department of Ophthalmology, Blesa University Hospital and the Instituto Aragones de Ciencias de la Salud, Zaragoza, Spain

Abstract

Corresponding authors: David M. Gamm, dgamm@wisc.edu, Phone: (608) 261-1516, Fax: (608) 890-3479. Jason S. Meyer, meyerjas@iupui.edu, Phone: (317) 274-1040, Fax: (317) 274-2846.

*Current Address: Department of Biology, Indiana University-Purdue University Indianapolis, 723 West Michigan Street, Indianapolis, IN 46202.

COMPETING INTERESTS STATEMENT

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Author Contributions

Jason S. Meyer: Conception and design, Collection and/or assembly of data, Data analysis and interpretation, Manuscript writing, Final approval of manuscript

Sara E. Howden: Conception and design, Collection and/or assembly of data, Data analysis and interpretation, Manuscript writing, Final approval of manuscript

Kyle A. Wallace: Collection and/or assembly of data, Data analysis and interpretation

Amelia D. Verhoeven: Collection and/or assembly of data

Lynda S. Wright: Data analysis and interpretation, Manuscript writing

Elizabeth E. Capowski: Conception and design, Data analysis and interpretation

Isabel Pinilla: Collection and/or assembly of data

Jessica M. Martin: Collection and/or assembly of data

Shulan Tian: Collection and/or assembly of data, Data analysis and interpretation, Manuscript writing

Ron Stewart: Collection and/or assembly of data, Data analysis and interpretation, Manuscript writing, Final approval of manuscript

Bikash Pattnaik: Conception and design, Financial support, Collection and/or assembly of data, Data analysis and interpretation, Manuscript writing, Final approval of manuscript

James Thomson: Conception and design, Financial support, Data analysis and interpretation, Final approval of manuscript

David M. Gamm: Conception and design, Financial support, Data analysis and interpretation, Manuscript writing, Final approval of manuscript

Differentiation methods for human induced pluripotent stem cells (hiPSCs) typically yield progeny from multiple tissue lineages, limiting their utility for drug testing and autologous cell transplantation. In particular, early retina and forebrain derivatives often intermingle in pluripotent stem cell cultures, owing to their shared ancestry and tightly coupled development. Here, we demonstrate that three-dimensional populations of retinal progenitor cells (RPCs) can be isolated from early forebrain populations in both human embryonic stem cell (hESC) and hiPSC cultures, providing a valuable tool for developmental, functional, and translational studies. Using our established protocol, we identified a transient population of optic vesicle-like (OV) structures that arose during a time period appropriate for normal human retinogenesis. These structures were independently cultured and analyzed to confirm their multipotent RPC status and capacity to produce physiologically responsive retinal cell types, including photoreceptors and retinal pigment epithelium (RPE). We then applied this method to hiPSCs derived from a patient with gyrate atrophy, a retinal degenerative disease affecting the RPE. RPE generated from these hiPSCs exhibited a disease-specific functional defect that could be corrected either by pharmacological means or following targeted gene repair. The production of OV-like populations from human pluripotent stem cells should facilitate the study of human retinal development and disease and advance the use of hiPSCs in personalized medicine.

Keywords

Pluripotent stem cells; Retina; Developmental Biology; Neural differentiation; Cellular therapy; iPS

INTRODUCTION

Human pluripotent stem cells, which include both human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs), hold the potential to differentiate into any cell type. In doing so, they can serve as comprehensive model systems of human cell genesis, particularly at early developmental stages that would otherwise be inaccessible to investigation^{1,2}. In addition, patient-derived hiPSC lines have a unique capacity to model human disease³⁻⁶, although the scope of disorders amenable to this form of study is limited⁷⁻⁹. Major considerations when creating hiPSC disease models include the capacity to efficiently generate, identify and isolate relevant cell populations, as well as recapitulate and assay critical aspects of the disease mechanism.

Retinal cell types are particularly well-suited for the investigation of cell development and dysfunction using pluripotent stem cell technology. The vertebrate retina harbors a modest repertoire of major cell classes sequentially produced via a conserved series of events¹⁰⁻¹⁶. Furthermore, the effects of inherited and acquired retinal degenerative diseases (RDD) are often limited initially to a specific cell class, which simplifies the study of cellular mechanisms that incite RDD and the evaluation of potential therapies.

Previous studies have demonstrated the ability of human pluripotent stem cells to differentiate along the retinal lineage with varying efficiencies¹⁷⁻²⁰, with one protocol achieving a near uniform retinal cell fate using the WA01 hESC line¹⁸. However, pluripotent stem cell-derived retinal cells, particularly those from hiPSCs, are most often found in mixed populations that include some non-retinal or unidentified cell types^{17,20-22}. Further complicating matters is the fact that several markers used for retinal cell identification (e.g., calretinin, PKC α , Tuj1) also label cells found in other regions of the CNS. As such, a means to isolate developmentally synchronized populations of multipotent retinal progenitor cells (RPCs) across multiple hESC and hiPSC lines would be desirable. The RPCs and their definitive retinal progeny could then be used to study mechanisms of

human retinal development and disease, examine retinal cell function, and devise and test RDD treatments.

We recently described a method to differentiate human pluripotent stem cells to RPCs, retinal pigment epithelium (RPE), and photoreceptor-like cells in a manner that mimicked normal human retinogenesis¹⁷. However, a means to separate and track the fate of the RPCs in live culture was not available. In the current study, we used transient morphological features to isolate structures with characteristics reminiscent of the optic vesicle (OV). Using these OV-like structures, it was possible to study principles of early human retinal development, monitor the sequence and timing of neuroretinal cell genesis, and optimize RPC and RPE production efficiencies in recalcitrant hiPSC lines. We then utilized our system to evaluate pharmacologic and genetic strategies to correct a functional defect in hiPSC-RPE cultures derived from a patient with an inherited RDD.

MATERIALS AND METHODS

Maintenance and differentiation of human pluripotent stem cells

hESCs (WA09 and WA01) and hiPSCs (IMR90-4²³, 6-9-12T²⁴, iPS-12²⁵, iPS-12.4²⁵, and three patient-specific lentivirus-derived hiPSC lines) were maintained in ESC medium (see Supporting Information Methods for media descriptions) using an established method^{17,23}. Differentiation toward anterior neuroectodermal and retinal fates was accomplished using a modification of a previously published protocol¹⁷. Briefly, human pluripotent stem cell colonies were cultured as suspended aggregates for 4 days in EB medium, whereupon they were switched to Neural Induction Medium. For some experiments, cultures were treated from day 2–4 with 100 ng/ml Noggin (or Dorsomorphin) and DKK1 (or XAV939). After a total of 6–7 days, aggregates were plated onto laminin-coated substrates to permit formation of neural clusters. On day 16, the loosely adherent central portions of the neural clusters were mechanically lifted and grown as cellular aggregates in Retinal Differentiation Medium (RDM). Between day 20–25, a subset of these aggregates adopted a vesicle-like appearance, which was manually separated and differentiated in RDM. Nonvesicular aggregates, or spheres, were likewise separated, pooled, and differentiated in RDM.

Generation of RPE from vesicle-like cultures was enhanced by adding Activin A (100 ng/ml) to RDM from day 20–40 of differentiation. RPE from manually isolated pigmented OV-like structures was expanded by plating them onto laminin-coated substrates in RDM + 20 ng/ml FGF2/EGF and 5 μ g/ml heparin for up to one week as described for prenatal RPE cultures²⁶. Large patches of RPE-like cells could then be manually isolated, dissociated, and re-plated in the presence of mitogens for at least one additional passage.

Immunocytochemistry

ICC analysis was carried out as described¹⁷. For a description of sectioning and immunostaining, see Supporting Information Methods. For a list of antibodies, see Supporting Information Table S1.

PCR analysis

RNA was isolated using the RNeasy (Qiagen) or Picopure RNA isolation kit (Arcturus), reverse transcribed, and amplified by PCR (30 cycles) or quantitative PCR (qPCR) (40 cycles) as described¹⁷. Primer sets are listed in Supporting Information Table S2. For a description of the microarray analysis method, see Supporting Information Methods.

Generation of patient-specific iPS cells

Gyrate atrophy fibroblasts were obtained from Coriell Institute for Medical Research (GM06330) and propagated in DMEM with 10% FBS. In addition, fibroblasts from two individuals without known genetic diseases were cultured from skin samples, as approved by the University of Wisconsin-Madison IRB. Reprogramming was performed as previously described²³ using lentiviral vectors expressing *OCT4*, *SOX2*, *NANOG*, *LIN28*, *c-MYC*, and *KLF4*.

Ornithine- δ -aminotransferase assay

OAT activity in RPE cultures was determined by the method of Ueda et al.²⁷, as detailed in Supporting Information Methods.

Electrophysiology and live cell imaging

OV-like structures were differentiated in suspension culture for a total of 100 days and plated onto poly-D-ornithine- and laminin-coated coverslips for an additional 2 days to allow for adherence and peripheral cell spread/migration. Ionic currents were measured using conventional whole cell recordings performed on a fixed stage of a Nikon FN-1 microscope. Cells were superfused continuously at ~2 ml/min in standard bath solution (see Supporting Information Methods). Recordings were acquired using patch pipettes (3–5 M Ω) filled with pipette solution (see Supporting Information Methods), an Axopatch 200B amplifier, Digidata 1440 interface and pCLAMP 10 software (Molecular Devices). Stored data was analyzed using Clampfit-10 and plotted using Excel and Origin-8. Current responses were generated using either a voltage ramp (–160 to +40 mV) or step (–160 to +40 mV in 10 mV steps) protocol from a holding potential of –70 mV. A junction potential of 11 mV was calculated using pCLAMP software and all records were compensated post hoc.

For Ca²⁺ imaging, cells were loaded with 5 μ M Fura-2 AM (Invitrogen) for 60 min at 37°C. After washing for 30 min, coverslips were placed at the bottom of the recording chamber. 1 mM probenecid was added to prevent dye extrusion, and cells were sequentially exposed to 340 and 380 nm excitation wavelengths while registering fluorescence emission at 510 nm. Images were collected every 10 s with 2 $\times$ 2 pixel binning using a cooled CCD camera (CoolSNAP HQ, Photometrics) and a 60X water immersion objective. NIS Elements was used for image acquisition and offline analysis to determine changes in [Ca²⁺]_i using the equation listed in Supporting Information Methods. Results were expressed as mean $\pm$ SEM.

RESULTS

Isolation of hESC structures with characteristics of the optic vesicle or early forebrain

The OV is derived from the primitive anterior neuroepithelium (PAN) and shares numerous features with the early forebrain^{28–30}. We previously demonstrated the ability to differentiate human pluripotent stem cells into PAN, which in turn gave rise to free-floating cell aggregates containing either RPCs or early forebrain cells¹⁷. In the present study, we looked for features that could be used to separate the RPC and early forebrain populations in live culture without the need for genetic manipulation.

Using our protocol¹⁷, densely packed, neural rosette-containing cell colonies were isolated intact from WA09 hESCs after 16 days of differentiation and cultured in suspension. Starting at 20 days, two populations of cell aggregates could be distinguished via light microscopy (Fig. 1A). A minority population (20.4 $\pm$ 4.3%) was phase-bright and appeared vesicle-like due to the presence of a compact outer mantle of cells. The remaining population was spherical and had a more uniform cell distribution, although internal rosettes

were commonly observed. Some cell aggregates possessed elements of both populations; however, these hybrid structures were excluded from this study.

Cell aggregates with an unequivocal vesicle-like or nonvesicular configuration were separated between day 20–25 and cultured independently (Fig. 1B,C). Because this period of differentiation corresponded developmentally to the formation of OV in humans, we asked whether CHX10, a marker of RPCs in the distal OV, was differentially expressed between the two populations. Prior to separation, CHX10 was found in a subset of cell aggregates (Fig. 1D). Afterward, CHX10 immunoreactivity was abundant ($90.88 \pm 4.23\%$ of total cells) in the vesicle-like structures (Fig. 1E) and nearly absent in the nonvesicular spheres (Fig. 1F). By contrast, ISLET-1, a homeodomain protein involved in projection neuron differentiation and early forebrain development, was found only in the nonvesicular population at this stage (Fig. 1G–I).

Given the disparate expression of CHX10 and ISLET-1 between the vesicle-like structures and nonvesicular spheres, PCR was performed to investigate gene expression patterns of other transcription factors involved in early retinal and/or forebrain development (Fig. 1J). After 20 days, the vesicle-like structures selectively expressed multiple retinal transcription factor genes appropriate for the OV stage of retinogenesis, whereas the nonvesicular spheres expressed transcription factors indicative of the embryonic forebrain. Factors involved in the early development of both the retina and forebrain were likewise present in both culture populations.

The progenitor state of the CHX10+ vesicle-like structures was further queried using the cell proliferation marker Ki67. ICC analysis of sections revealed coexpression of CHX10 and Ki67 and confirmed the compact, radial arrangement of nuclei within these structures (Fig. 1K). With further maturation, they often lost their distinctive morphology and developed internal rosettes, rendering them indistinguishable from the nonvesicular spheres (Fig. 1L). However, most cells remained CHX10+ and/or Ki67+ until approximately 50 days of differentiation (Fig. 1M), after which time the expression of these markers progressively declined in lieu of more mature retinal markers (see Fig. 2).

We next performed comparative gene microarray analysis to further highlight differences in transcription factor gene expression between day 20 vesicle-like structures and nonvesicular spheres (Supporting Information Fig. S1). Many transcription factor genes associated with retinal development were present at higher levels in the vesicle-like structures, including *SIX6*, *RAX*, *MITF*, *TBX2*, *TBX5*, and *VAX2*. Nonvesicular spheres, on the other hand, expressed higher levels of transcription factors implicated in early forebrain or general neural development, including *DLX1*, *DLX2*, *SOX1*, and *ISLET-1*. Taken together, results from the comparative ICC, PCR, and microarray analyses indicated that the vesicle-like structures and nonvesicular spheres harbored RPC and early forebrain populations, respectively. Furthermore, the gene and protein expression profiles of the RPCs reflected a differentiation state akin to the OV stage of retinal development. Therefore, the vesicle-like structures and nonvesicular spheres were re-designated OV-like structures and early forebrain (EFB) spheres, respectively.

Longitudinal differentiation analysis of OV-like structures and EFB spheres

Many markers used to distinguish non-photoreceptor cell types within the neuroretina are found elsewhere in the CNS, compromising efforts to study retinogenesis in mixed lineage pluripotent stem cell cultures. To circumvent this issue, we performed longitudinal differentiation analyses on isolated populations of OV-like structures and EFB spheres. Because OV-like structures contained a nearly exclusive population of RPCs, differentiated

progeny from these cultures could be assigned to the retinal lineage with a high degree of certainty.

Cultures of WA09 hESC OV-like structures were sampled every ten days from day 20–120 of differentiation and analyzed by qPCR (Fig. 2A). *BRN3* and *CRX*, neuroretinal markers of retinal ganglion cells (RGCs) or early post-mitotic photoreceptors, respectively, were among the first genes to be expressed. The *CALRETININ* gene (amacrine cells, horizontal cells, and some RGCs) was expressed at a nearly constant level after day 40. Later-expressed genes included *NRL* (post-mitotic rod precursors) and *PKC-α* (bipolar cells). The presence of multiple neuroretinal cell types was confirmed at day 80 by ICC using 1° antibodies against BRN3 and βIII-TUBULIN (Fig. 2B), CALRETININ (Fig. 2C), PKC-α and CHX10 (Fig. 2D), CRX and RECOVERIN (Fig. 2E–G), and NRL (Fig. 2H). Cells expressing photoreceptor markers often displayed a distinct morphology, bearing one thin process opposite a thicker, tubular or conical process (Fig. 2F,G). ICC quantification revealed that most progeny of OV-like structures produced by day 80 expressed photoreceptor or RGC markers (CRX: $55.9 \pm 6.6\%$; BRN3: $14.6 \pm 3.2\%$ of all cells). Immunostained sections of OV-like structures showed segregation of retinal cell populations, with most BRN3+ cells concentrated near the periphery, whereas RECOVERIN+ cells tended to congregate internally in clusters or linear configurations (Supporting Information Fig. S2).

EFB spheres maintained an anterior neural identity upon further differentiation, generating a variety of phenotypes found within the forebrain. At 20 days, EFB spheres expressed PAX6 (Fig. 3A), SOX1 (Fig. 3B), OTX2 (Fig. 3C), and βIII TUBULIN (Fig. 3A–C). By day 70, EFB spheres yielded more mature cell phenotypes, including GABAergic neurons (Fig. 3D), tyrosine hydroxylase (TH)+ dopaminergic neurons (Fig. 3E), and GFAP+ astrocytes (Fig. 3F). No retina-specific markers were present in EFB spheres at any time point tested (up to day 70). The anterior neural character of day 20 EFB spheres was further confirmed by PCR, which revealed expression of forebrain, but not midbrain (*EN-1*) or hindbrain (*HOXB4*) genes (Fig. 3G). The expression of some of these genes was maintained at day 70 (e.g., *FOXP1*), while others were down- (e.g., *PAX6*, *OTX2*) or up- (e.g., *MAP2*, *TH*) regulated.

Photoreceptor-like cells derived from OV-like structures display characteristic electrophysiological responses

Examination of electrophysiological properties offers a powerful means of evaluating both cell identity and functional capacity. WA09 hESC OV-like structures were differentiated for 100 days and plated on coverslips, whereupon cells with photoreceptor-like morphology were recorded using standard patch clamp techniques. To verify identity, recorded cells were loaded with sulphorhodamine (Fig. 4A) and later immunostained for RECOVERIN (Fig. 4B). PCR analysis of these cultures confirmed expression of genes associated with phototransduction, including the cyclic nucleotide-gated channel subunits *CNGA1*, *A3*, *B1*, and *B3*, the guanylyl cyclase gene *RETGC*, the cGMP phosphodiesterase gene *PDE6B*, and *ARRESTIN* (Fig. 4C). RECOVERIN+ cells produced an outward positive current that was activated at depolarizing voltages between –50 and +40 mV from a holding potential of –70 mV (Fig. 4D). Upon achieving whole-cell configuration, these cells registered a resting membrane potential of -44 ± 4 mV and a current at +40 mV of 27 ± 8 pA/pF ($n = 15$), compared to -29 ± 2 mV and 9 ± 1 pA/pF for control, non-photoreceptor cells ($n=3$) (Fig. 4D). The current-voltage (I-V) plot revealed a large outward current with a linear I-V relationship between –10 to +40 mV, but no inward current. The voltage-dependent outward current was suppressed with 15 mM tetraethylammonium (TEA), and measurements at both +20 and +40 mV showed that the TEA-sensitive component possessed fast activation kinetics without deactivation during the 500 ms voltage pulse (Fig. 4E). I-V curves confirmed the selective reduction of outward current by external TEA from an average of

468 ± 139 pA to 89 ± 25 pA (measured at +40 mV; n=5 cells) (Fig. 4F). Given the low [Ca²⁺] pipette solution used for these experiments and the TEA sensitivity of the current, we concluded that delayed rectifier potassium channels were responsible for the observed voltage-dependent outward current, consistent with photoreceptor electrophysiology^{31–35}.

In the dark, photoreceptors are maintained in a depolarized state via influx of Ca²⁺ and Na⁺ through nonselective cGMP-gated plasma membrane cation channels^{36–38}. Light-stimulated hydrolysis of cGMP results in closure of these channels and membrane hyperpolarization³⁹. To further test the functional identity of the photoreceptor-like cells, we exposed them to membrane-permeable 8-br-cGMP, which resulted in an instantaneous increase in inward current amplitude of >4-fold (measured at –70 mV holding potential) (Fig. 4G), consistent with the known kinetics of cyclic nucleotide-gated ion channels⁴⁰. A similar sustained increase in current amplitude was observed at depolarized membrane potentials (Fig. 4H,I). Overall, exposure to 8-br-cGMP converted the outward rectifying I-V plot to a linear I-V plot that crossed close to 0 mV (Fig. 4I), indicating a switch to nonselective ion conductance⁴⁰. Furthermore, 8-br-cGMP treatment resulted in membrane depolarization from –44 ± 4 mV to –7.7 ± 1.8 mV, similar to the difference between the light and dark resting membrane potentials of photoreceptors³⁹. These features are found solely in cells that undergo phototransduction^{31,39}, and provide further evidence that RECOVERIN+ cells generated from these cultures possess an electrophysiological signature highly reminiscent of photoreceptors.

OV-like structures can be directed to an RPE fate

RPE was rarely observed when OV spheres were cultured in isolation, regardless of the duration of differentiation. Previous reports demonstrated that the TGFβ superfamily member Activin A can promote an RPE fate at the expense of neuroretina^{41,42}. To test whether a similar effect could occur in our cultures, 100 mg/ml Activin A was added to cultures of OV-like structures from day 20–40. Activin A-treated cultures produced subsets of structures with deep pigmentation beginning between day 40–60 (Fig. 5A,B). Pigmented OV-like structures were manually separated from non-pigmented OV-like structures (Fig. 5C) and adhered to substrate in the presence of mitogens to promote cell proliferation. Under these conditions, cells proliferated and formed monolayers (Fig. 5D). Removal of mitogens resulted in reestablishment of cellular pigmentation, polygonal morphology (Fig. 5E), and gene and protein expression patterns characteristic of RPE (Fig. 5F; Supporting Information Fig. S3). qPCR analysis (Fig. 5G,H) revealed a reciprocal influence of Activin A on the expression of *MITF* (8.3 ± 2.5-fold increase) and *CHX10* (5.3 ± 1.4-fold decrease), transcription factors associated with the development of RPE and neuroretina, respectively. Activin A-treated cultures also expressed RPE genes such as *RPE65* and *BEST1* at higher levels (4.9 ± 1.8- and 19.1 ± 5.4-fold, respectively) than untreated OV-like structures (Fig. 5G), and lower levels of genes associated with neuroretina, including *CRX* and *BRN3B* (2.4 ± 0.6- and 1.9 ± 0.4-fold, respectively) (Fig. 5H).

To assay the functional potential of hESC-RPE cells, we chose to examine their response to ATP, a molecule postulated to govern light-induced activation of purinergic signaling pathways, leading to intracellular Ca²⁺ mobilization and directional fluid transport across the RPE⁴³. hESC-RPE cells exhibited an increase in [Ca²⁺]_i (58 ± 4.5 nM) immediately after exposure to 100 μM ATP, followed by a decline to steady state levels (Fig. 5I–K). These responses were comparable to those of prenatal human RPE cultures (Supporting Information Fig. S3). Therefore, similar to other human pluripotent stem cell differentiation protocols^{42,44–46}, RPE derived with our method displayed important physiological properties that can be exploited to study cell function and test therapeutics.

Production of OV-like structures from human iPS cells

Lineage-specific differentiation efficiencies vary across hiPSC lines, which restricts their utility^{7,17,45,47}. A better understanding of the factors governing hiPSC differentiation potential may help optimize methods for the targeted production of neural and retinal cell types. We screened four hiPSC and two hESC lines via qPCR for expression of selected genes involved in early neuro- and retinogenesis. Significant variability was found in the expression levels of anterior neural/eye field genes across these lines at day 10 (Fig. 6A,B; Supporting Information Fig. S4). Lines displaying the lowest levels of these genes mainly produced flat, epithelioid colonies (Fig. 6C) that lacked expression of anterior neural/eye field markers (Fig. 6D).

We next investigated very early gene expression levels of *DKK1* and *NOGGIN*, two pro-anterior neural/eye field factors that antagonize the canonical Wnt and BMP signaling pathways, respectively^{20,22}. Prolonged addition of one or both of these factors is common to human pluripotent stem cell neural and retinal differentiation protocols^{20,22}; however, it is unclear whether endogenous *DKK1* and *NOGGIN* expression levels predict competency to produce anterior neural progeny. We found that hiPSC lines expressing the highest levels of *DKK1* and, to a lesser extent, *NOGGIN* at day 2 (Fig. 6E,F) also expressed the highest relative levels of anterior neural/eye field genes at day 10 (Fig. 6A,B; Supporting Information Fig. S4) and CHX10+ OV-like structures at day 20 (Fig. 6G,H). OV-like structures isolated from hiPSCs were indistinguishable from those derived from hESCs and, upon further differentiation, generated neuroretinal cell types (Supporting Information Fig. S5). RPE produced from hiPSC cultures was also similar to human prenatal and hESC-derived RPE based on appearance, gene and protein expression, and physiological response to ATP (Supporting Information Fig. S3).

Given the apparent correlation between early endogenous *DKK1* and *NOGGIN* expression and the ensuing acquisition of an anterior neural/eye field fate, we hypothesized that *DKK1* and *NOGGIN* (or their small molecule equivalents) need only be added during a short developmental time window to augment neural and retinal specification in recalcitrant hiPSC lines. To test this theory, *DKK1* (or XAV-939) and *NOGGIN* (or Dorsomorphin) were added to our lowest anterior neural/eye field gene-expressing hiPSC line (Lenti iPSC #2) from day 2–4 and analyzed by qPCR six days later. Treated cultures expressed 84.8 ± 1.9 - and 156.3 ± 59.5 -fold higher levels of *PAX6* and *RAX*, respectively, compared to untreated cultures (Fig. 6I). Morphologically, treated hiPSCs produced mounded colonies of neuroepithelial cells at day 10 (Fig. 6J) that expressed anterior neural/eye field transcription factors (Fig. 6K). The neuralization of treated hiPSCs was further confirmed through FACS analysis, which revealed a 2.7-fold increase in the percentage of *PAX6*+ cells at day 10 (72.6 vs. 27.3%; Fig. 6L) and a 6.7-fold increase in the percentage of CHX10+ cells at day 20 (34.1 vs. 5.1%; Fig. 6M) compared to untreated cultures.

OV-like structures from patient-specific iPS cells can be used to test pharmacologic and genetic approaches to retinal disease treatment

A reliable means of isolating RPCs from patient-specific iPSCs would facilitate the study and treatment of retinal diseases⁴. Gyrate atrophy (GA) is a rare autosomal recessive RDD that primarily affects RPE, causing secondary photoreceptor loss and blindness^{27,48–50}. GA is caused by a wide range of defects in the gene encoding ornithine- δ -aminotransferase (OAT), a vitamin B₆-dependent enzyme that catalyzes the interconversion of L-ornithine and Δ^1 -pyrroline-5-carboxylate⁴⁸. hiPSCs were derived from fibroblasts of a GA patient bearing the A226V OAT mutation (Fig. 7A,B) and screened for expression of pluripotency genes, ability to form teratomas, retention of the *OAT* gene mutation, and maintenance of a normal karyotype (Supporting Information Fig. S6).

Using our protocol, (A226V)OAT hiPSCs formed neuroepithelial structures (Fig. 7C) and expressed anterior neural/eye field markers at day 10 (Fig. 7D). OV-like structures isolated from these cultures generated neuroretinal progeny (Fig. 7E), but rarely became pigmented in the absence of Activin A. OAT activity in RPE derived from (A226V)OAT hiPSCs (Fig. 7F) was then measured using a colorimetric assay²⁷. In the presence of 50 μ M vitamin B₆, (A226V)OAT hiPSC-RPE had very low OAT activity (5.9 ± 3.0 μ mol/hr/ 10^6 cells; n=3) (Fig. 7G) compared to control RPE cultures derived from IMR90-4 hiPSCs, WA09 hESCs, and prenatal human eyes (68.9 ± 9.9 , 142.7 ± 19.7 , and 91.2 ± 10.0 μ mol/hr/ 10^6 cells, respectively).

Because the particular mutation in this GA patient is postulated to affect vitamin B₆ binding to the enzyme⁵¹, we next investigated whether elevated levels of vitamin B₆ could restore OAT activity. Increasing vitamin B₆ to 200 μ M was ineffective; however, 600 μ M vitamin B₆ greatly enhanced enzymatic activity (24.5 ± 9.8 -fold; n=3) in (A226V)OAT hiPSC-RPE (Fig. 7H). In contrast, 600 μ M vitamin B₆ only increased OAT activity in control RPE cultures by 1.7 ± 0.1 -fold (n=3) and in (A226V)OAT fibroblasts by 8.1 ± 3.2 -fold (n=5). Higher levels of vitamin B₆ (1200 μ M) failed to increase OAT activity further.

Lastly, we applied our differentiation protocol to a gyrate atrophy hiPSC line (iPS-12.4) that previously underwent repair of the *OAT* gene mutation via bacterial artificial chromosome (BAC)-mediated homologous recombination²⁵. Contrary to RPE derived from the (A226V)OAT parent line (iPS-12), the gene-corrected hiPSC-RPE displayed normal levels of OAT activity (130.0 ± 12.4 μ mol/hr/ 10^6 cells; n=3) (Fig. 7I). Therefore, OV-like structures provide a practical and versatile intermediary in the production of retinal cell types from hiPSCs.

DISCUSSION

The ability to isolate tissue-specific progenitor populations from live, differentiating human pluripotent stem cells enhances their scientific and clinical utility. Beyond supplying a potentially expandable and relatively uniform cell source for transplantation, such populations facilitate in vitro studies of human cellular development and disease.

The development of the vertebrate retina can be traced morphologically, beginning with the evagination of OVs from the lateral aspects of the primitive forebrain. At this stage, chick OVs can be dissected from surrounding tissues and cultured short-term as explants, during which time they maintain their characteristic vesicular structure⁴¹. However, a similar in vitro hallmark of early retinal populations had not been described in human pluripotent stem cell cultures prior to this report. OV-like structures isolated from hESCs and hiPSCs were anterior neuroectodermal in origin, expressed early retinal markers, and gave rise to mature retinal cell types, confirming their status as RPCs. In addition, the order of appearance of retinal cell types from OV-like structures approximated the conserved cell birth order found during vertebrate retinogenesis^{10,12}.

While OV-like structures resemble true OVs in some respects, the similarities between them are limited. Unlike true OVs, hESC- and hiPSC-derived OV-like structures did not invaginate to form bilayered cups using the minimal culture conditions we employed. Instead, they “filled in” and became relatively unorganized, presumably as a result of continued RPC proliferation in the absence of appropriate signaling cues. In addition, during normal vertebrate OV development, production of Chx10+ neuroretinal progenitors is restricted to the distal OV, whereas the proximal OV remains Mitf+ and yields RPE^{29,30}. This segregation of the neuroretinal and RPE domains is governed in part by reciprocal effects of FGFs and TGF β superfamily members produced by surface ectoderm and

extraocular mesenchyme, respectively^{41,52}. Similarly, canonical Wnt signaling has been shown to promote RPE generation while antagonizing formation of neuroretina⁵³. The lack of RPE production in isolated OV-like structures, which are cultured in defined medium without these factors, suggests an intrinsic bias toward a neuroretinal fate. In support of this hypothesis, we found that hESC-derived sphere cultures endogenously expressed FGFs along with inhibitors of TGF β superfamily and Wnt signaling¹⁷. In the present study, we further showed that this neuroretinal bias could be partially overcome via the addition of the TGF β superfamily member Activin A. An analogous situation has been described in the chick model, where OV explants grown in isolation failed to generate RPE, but did so when exposed to Activin A⁴¹.

Beyond adopting distinct morphologies and expression profiles, we also demonstrated that retinal cells derived from human pluripotent stem cells possessed important functional properties. Patch clamp recordings of RECOVERIN+ cells revealed a current-voltage relationship consistent with mammalian photoreceptors^{31–35}, as well as robust depolarization in response to administration of cGMP analogs. The latter finding is particularly intriguing, since cGMP is a critical second messenger of the phototransduction cascade³⁸, responsible for modulating the circulating 'dark current'³⁹. While RGCs can also depolarize after cGMP stimulation⁵⁴, all cGMP-responsive cells tested in our cultures were RECOVERIN+ and lacked long neurite projections characteristic of RGCs. Therefore, the identity of the recorded cells was most consistent with photoreceptors. To document functionality of hESC- and hiPSC-RPE cells in a novel manner, we examined their response to ATP, an extracellular signaling molecule linked to the generation of IP₃ and release of intracellular calcium⁵⁵. Through this mechanism, ATP is proposed to regulate ion and fluid flux across the RPE⁴³, among other critical functions. Exposure of hESC- and hiPSC-RPE to ATP resulted in rapid and reversible increases in intracellular calcium, analogous to responses elicited from prenatal human RPE cultures.

While the isolation of OV-like structures from differentiating hESCs provides a dynamic tool for the study of human retinal development, it may prove more useful for hiPSC research. Multiple reports have detailed variability in fate potential between hiPSC lines^{7,17,45,47}, some of which displayed little capacity to yield retinal cell types. In the present study, we found that OV-like structures were generated by every differentiating hiPSC line tested, although sometimes in very low abundance. Even so, their distinctive appearance allowed them to be isolated and cultured independently. It was also noted that retinal differentiation efficiency across hiPSC lines correlated with early endogenous expression of factors (DKK1 and NOGGIN) known to influence anterior neural fate specification. We have since used this information to quickly and inexpensively screen new hiPSC lines for competency to produce OV-like structures.

As methods of hiPSC production and differentiation continue to improve, so will the potential to apply this technology to model and treat human diseases. Inherited RDDs appear particularly amenable to hiPSC modeling, since many exhibit gene-specific, cell autonomous features that can be tested in vitro. In addition, broad sequelae common to multiple inherited RDDs, such as loss of rods, can be monitored in culture and used to assess drug efficacy⁵. For the present study, we chose to derive hiPSCs from a patient with GA, an RPE-based RDD for which a simple assay exists to measure activity of the affected gene product, OAT²⁷. This enzyme is expressed in multiple tissues, but has particularly high activity in RPE²⁷, which may explain why clinical manifestations of GA are largely restricted to the retina. We confirmed that (A226V)OAT hiPSC-RPE had very low OAT activity, and further showed that these cells were exceptionally responsive to treatment with vitamin B₆, a pharmacological agent known to reduce serum ornithine levels and improve fibroblast OAT activity in a small cohort of GA patients. However, direct tests of vitamin B₆

efficacy in RPE derived from a living GA patient have heretofore been unfeasible. We found that 600 μ M vitamin B₆, but not 200 μ M, completely restored OAT activity in (A226V)OAT hiPSC-RPE, while higher concentrations showed no additional benefit. These findings are potentially important since excessive vitamin B₆ supplementation can cause painful and ultimately irreversible neurological side effects⁵⁶.

Perhaps the most imminent clinical role of hiPSC technology is in pharmacologic testing of rare, genetically heterogeneous diseases that 1) display variations in drug efficacy between individuals and 2) affect tissues that cannot be routinely or safely biopsied. Even when surrogate tests of drug response are available, patients may be better served by direct testing of cell types targeted by the disease. For example, information from the repository where we obtained the GA fibroblasts indicated that the donor was vitamin B₆ unresponsive. However, other patients with the A226V OAT mutation⁵¹, as well as the donor's own hiPSC-RPE, were shown to respond to such treatment. It is therefore possible that the patient would have had a therapeutic response to vitamin B₆ at the RPE level, but because efficacy was determined by less sensitive means, the treatment was erroneously deemed unbeneficial.

A more distant application of hiPSCs may be as a source of autologous donor cells for replacement therapies. To serve in this capacity, repair of disease-causing gene defects will likely be necessary. Recently, Howden et al. corrected the *OAT* mutation in our patient using BAC-mediated homologous recombination²⁵ and showed that this process did not introduce new mutations into the hiPSC genome. Here, we further demonstrated that OAT activity was fully restored in the gene-corrected hiPSC-RPE. To our knowledge, this is the first report of functional correction of disease-specific hiPSCs using both pharmacologic and genetic approaches.

CONCLUSION

We demonstrated that vesicle-like structures corresponding to the OV stage of retinal development could be manually isolated from hESC and hiPSC lines. The production of highly enriched populations of physiologically active retinal cell types from hiPSCs provides a platform to investigate patient-specific drug effects, gene repair strategies, and disease mechanisms in vitro. However, caution must be exercised when extrapolating data obtained from cell culture systems to whole organisms. As such, hiPSC technology is envisioned to complement, not supplant, existing laboratory models of disease.

ADDENDUM

While our manuscript was under review, Eiraku et al.⁵⁷ published a report demonstrating that mouse ESCs could form OV structures, which subsequently self-organized into bilayered optic cups that produced both RPE and laminated neuroretinal tissue. Interestingly, when the mouse hESC-derived OVs were separated from attached non-retinal neuroectoderm and cultured in isolation, they failed to form optic cups and became biased to a Chx10+ neuroretinal fate, similar to our findings with isolated human ESC- and iPSC-derived OV-like structures. In light of our results and those of Eiraku et al.⁵⁷, we suspect that OV-like structures from human ESCs and iPSCs are also capable of higher order structure and organization under certain culture conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Keller G. Embryonic stem cell differentiation: emergence of a new era in biology and medicine. *Genes Dev.* 2005; 19:1129–1155. [PubMed: 15905405]
2. Pera MF, Trounson AO. Human embryonic stem cells: prospects for development. *Development.* 2004; 131:5515–5525. [PubMed: 15509763]
3. Ebert AD, Yu J, Rose FF Jr, et al. Induced pluripotent stem cells from a spinal muscular atrophy patient. *Nature.* 2009; 457:277–280. [PubMed: 19098894]
4. Gamm DM, Meyer JS. Directed differentiation of human induced pluripotent stem cells: a retina perspective. *Regen Med.* 2010; 5:315–317. [PubMed: 20455642]
5. Jin ZB, Okamoto S, Osakada F, et al. Modeling retinal degeneration using patient-specific induced pluripotent stem cells. *PLoS One.* 2011; 6:e17084. [PubMed: 21347327]
6. Park IH, Arora N, Huo H, et al. Disease-specific induced pluripotent stem cells. *Cell.* 2008; 134:877–886. [PubMed: 18691744]
7. Belmonte JC, Ellis J, Hochedlinger K, et al. Induced pluripotent stem cells and reprogramming: seeing the science through the hype. *Nat Rev Genet.* 2009; 10:878–883. [PubMed: 19859062]
8. Stadtfeld M, Hochedlinger K. Induced pluripotency: history, mechanisms, and applications. *Genes Dev.* 2010; 24:2239–2263. [PubMed: 20952534]
9. Hanna JH, Saha K, Jaenisch R. Pluripotency and cellular reprogramming: facts, hypotheses, unresolved issues. *Cell.* 2010; 143:508–525. [PubMed: 21074044]
10. Barishak, Y. *Embryology of the Eye and its Adnexa.* New York: Karger; 2001.
11. Chow RL, Lang RA. Early eye development in vertebrates. *Annu Rev Cell Dev Biol.* 2001; 17:255–296. [PubMed: 11687490]
12. Finlay BL. The developing and evolving retina: using time to organize form. *Brain Res.* 2008; 1192:5–16. [PubMed: 17692298]
13. Hatakeyama J, Kageyama R. Retinal cell fate determination and bHLH factors. *Semin Cell Dev Biol.* 2004; 15:83–89. [PubMed: 15036211]
14. Marquardt T, Gruss P. Generating neuronal diversity in the retina: one for nearly all. *Trends Neurosci.* 2002; 25:32–38. [PubMed: 11801336]
15. Oliver G, Gruss P. Current views on eye development. *Trends Neurosci.* 1997; 20:415–421. [PubMed: 9292971]
16. Zhang SS, Fu XY, Barnstable CJ. Molecular aspects of vertebrate retinal development. *Mol Neurobiol.* 2002; 26:137–152. [PubMed: 12428752]
17. Meyer JS, Shearer RL, Capowski EE, et al. Modeling early retinal development with human embryonic and induced pluripotent stem cells. *Proc Natl Acad Sci U S A.* 2009; 106:16698–16703. [PubMed: 19706890]
18. Lamba DA, Reh TA. Microarray Characterization of Human Embryonic Stem Cell-Derived Retinal Cultures. *Invest Ophthalmol Vis Sci.* 2011
19. Lamba DA, McUsic A, Hirata RK, et al. Generation, purification and transplantation of photoreceptors derived from human induced pluripotent stem cells. *PLoS One.* 2010; 5:e8763. [PubMed: 20098701]
20. Osakada F, Ikeda H, Mandai M, et al. Toward the generation of rod and cone photoreceptors from mouse, monkey and human embryonic stem cells. *Nat Biotechnol.* 2008; 26:215–224. [PubMed: 18246062]

21. Banin E, Obolensky A, Idelson M, et al. Retinal incorporation and differentiation of neural precursors derived from human embryonic stem cells. *Stem Cells*. 2006; 24:246–257. [PubMed: 16123388]
22. Lamba DA, Karl MO, Ware CB, et al. Efficient generation of retinal progenitor cells from human embryonic stem cells. *Proc Natl Acad Sci U S A*. 2006; 103:12769–12774. [PubMed: 16908856]
23. Yu J, Vodyanik MA, Smuga-Otto K, et al. Induced pluripotent stem cell lines derived from human somatic cells. *Science*. 2007; 318:1917–1920. [PubMed: 18029452]
24. Yu J, Hu K, Smuga-Otto K, et al. Human Induced Pluripotent Stem Cells Free of Vector and Transgene Sequences. *Science*. 2009; 324:797–801. [PubMed: 19325077]
25. Howden SE, Gore A, Li Z, et al. Genetic correction and analysis of induced pluripotent stem cells from a patient with gyrate atrophy. *Proc Natl Acad Sci U S A*. 2011 in press.
26. Gamm DM, Melvan JN, Shearer RL, et al. A novel serum-free method for culturing human prenatal retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci*. 2008; 49:788–799. [PubMed: 18235029]
27. Ueda M, Masu Y, Ando A, et al. Prevention of ornithine cytotoxicity by proline in human retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci*. 1998; 39:820–827. [PubMed: 9538890]
28. Bharti K, Liu W, Csermely T, et al. Alternative promoter use in eye development: the complex role and regulation of the transcription factor MITF. *Development*. 2008; 135:1169–1178. [PubMed: 18272592]
29. Horsford DJ, Nguyen MT, Sellar GC, et al. Chx10 repression of Mitf is required for the maintenance of mammalian neuroretinal identity. *Development*. 2005; 132:177–187. [PubMed: 15576400]
30. Rowan S, Chen CM, Young TL, et al. Transdifferentiation of the retina into pigmented cells in ocular retardation mice defines a new function of the homeodomain gene Chx10. *Development*. 2004; 131:5139–5152. [PubMed: 15459106]
31. Balse E, Tessier LH, Forster V, et al. Glycine receptors in a population of adult mammalian cones. *J Physiol*. 2006; 571:391–401. [PubMed: 16396929]
32. Balse E, Tessier LH, Fuchs C, et al. Purification of mammalian cone photoreceptors by lectin panning and the enhancement of their survival in glia-conditioned medium. *Invest Ophthalmol Vis Sci*. 2005; 46:367–374. [PubMed: 15623797]
33. Kawai F, Horiguchi M, Ichinose H, et al. Suppression by an h current of spontaneous Na⁺ action potentials in human cone and rod photoreceptors. *Invest Ophthalmol Vis Sci*. 2005; 46:390–397. [PubMed: 15623800]
34. Pattnaik B, Jellali A, Sahel J, et al. GABAC receptors are localized with microtubule-associated protein 1B in mammalian cone photoreceptors. *J Neurosci*. 2000; 20:6789–6796. [PubMed: 10995822]
35. Picaud S, Pattnaik B, Hicks D, et al. GABAA and GABAC receptors in adult porcine cones: evidence from a photoreceptor-glia co-culture model. *J Physiol*. 1998; 513(Pt 1):33–42. [PubMed: 9782157]
36. Picones A, Korenbrot JJ. Permeation and interaction of monovalent cations with the cGMP-gated channel of cone photoreceptors. *J Gen Physiol*. 1992; 100:647–673. [PubMed: 1334122]
37. Yau KW. Phototransduction mechanism in retinal rods and cones. The Friedenwald Lecture. *Invest Ophthalmol Vis Sci*. 1994; 35:9–32. [PubMed: 7507907]
38. Yau KW, Baylor DA. Cyclic GMP-activated conductance of retinal photoreceptor cells. *Annu Rev Neurosci*. 1989; 12:289–327. [PubMed: 2467600]
39. Yau KW, Hardie RC. Phototransduction motifs and variations. *Cell*. 2009; 139:246–264. [PubMed: 19837030]
40. Menini A. Cyclic nucleotide-gated channels in visual and olfactory transduction. *Biophys Chem*. 1995; 55:185–196. [PubMed: 7542935]
41. Fuhrmann S, Levine EM, Reh TA. Extraocular mesenchyme patterns the optic vesicle during early eye development in the embryonic chick. *Development*. 2000; 127:4599–4609. [PubMed: 11023863]

42. Idelson M, Alper R, Obolensky A, et al. Directed differentiation of human embryonic stem cells into functional retinal pigment epithelium cells. *Cell Stem Cell*. 2009; 5:396–408. [PubMed: 19796620]
43. Peterson WM, Meggyesy C, Yu K, et al. Extracellular ATP activates calcium signaling, ion, and fluid transport in retinal pigment epithelium. *J Neurosci*. 1997; 17:2324–2337. [PubMed: 9065493]
44. Buchholz DE, Hikita ST, Rowland TJ, et al. Derivation of functional retinal pigmented epithelium from induced pluripotent stem cells. *Stem Cells*. 2009; 27:2427–2434. [PubMed: 19658190]
45. Liao JL, Yu J, Huang K, et al. Molecular signature of primary retinal pigment epithelium and stem-cell-derived RPE cells. *Hum Mol Genet*. 2010; 19:4229–4238. [PubMed: 20709808]
46. Klimanskaya I, Hipp J, Rezai KA, et al. Derivation and comparative assessment of retinal pigment epithelium from human embryonic stem cells using transcriptomics. *Cloning Stem Cells*. 2004; 6:217–245. [PubMed: 15671670]
47. Hu BY, Weick JP, Yu J, et al. Neural differentiation of human induced pluripotent stem cells follows developmental principles but with variable potency. *Proc Natl Acad Sci U S A*. 2010; 107:4335–4340. [PubMed: 20160098]
48. Valle, D.; Simell, O. The hyperornithinemias. In: Scriver, C.; Beaudet, A.; Sly, W.; Valle, D., editors. *The Metabolic and Molecular Bases of Inherited Disease*. 7. New York: McGraw-Hill; 1995. p. 1147-1185.
49. Wang T, Milam AH, Steel G, et al. A mouse model of gyrate atrophy of the choroid and retina. Early retinal pigment epithelium damage and progressive retinal degeneration. *J Clin Invest*. 1996; 97:2753–2762. [PubMed: 8675686]
50. Wang T, Steel G, Milam AH, et al. Correction of ornithine accumulation prevents retinal degeneration in a mouse model of gyrate atrophy of the choroid and retina. *Proc Natl Acad Sci U S A*. 2000; 97:1224–1229. [PubMed: 10655512]
51. Michaud J, Thompson GN, Brody LC, et al. Pyridoxine-responsive gyrate atrophy of the choroid and retina: clinical and biochemical correlates of the mutation A226V. *Am J Hum Genet*. 1995; 56:616–622. [PubMed: 7887415]
52. Nguyen M, Arnheiter H. Signaling and transcriptional regulation in early mammalian eye development: a link between FGF and MITF. *Development*. 2000; 127:3581–3591. [PubMed: 10903182]
53. Fujimura N, Taketo MM, Mori M, et al. Spatial and temporal regulation of Wnt/beta-catenin signaling is essential for development of the retinal pigment epithelium. *Dev Biol*. 2009; 334:31–45. [PubMed: 19596317]
54. Ahmad I, Leinders-Zufall T, Kocsis JD, et al. Retinal ganglion cells express a cGMP-gated cation conductance activatable by nitric oxide donors. *Neuron*. 1994; 12:155–165. [PubMed: 7507337]
55. Berridge MJ. Elementary and global aspects of calcium signalling. *J Physiol*. 1997; 499 (Pt 2): 291–306. [PubMed: 9080360]
56. Schaumberg, HH.; Berger, A. Pyridoxine neurotoxicity. In: Leklen, JE.; Reynolds, RD., editors. *Clinical and Physiological Applications of Vitamin B6*. New York: Alan R. Liss; 1988. p. 403-413.
57. Eiraku M, Takata N, Ishibashi H, et al. Self-organizing optic-cup morphogenesis in three-dimensional culture. *Nature*. 2011; 472:51–56. [PubMed: 21475194]

Fig. 1. Isolation of optic vesicle-like structures from hESCs

Between day 20–25 of hESC differentiation, free-floating cell aggregates displayed two morphologies: phase-bright vesicle-like structures (*arrows*) or darker, rosetted spheres (*arrowheads*) (**A**). These populations were manually separated into vesicle-like (**B**) and nonvesicular (**C**) pools and independently cultured (*arrows* indicate rosettes in nonvesicular spheres). The definitive retinal progenitor marker CHX10 was expressed exclusively in the population of vesicle-like structures (**D–F**), whereas ISLET-1 was initially expressed solely in the nonvesicular sphere population (**G–I**). PCR analysis identified optic vesicle (*left*) and early forebrain (*middle*) transcription factors that were differentially expressed at day 20–25 in vesicle-like structures (Vesc) and nonvesicular spheres (Non Vesc), as well as factors that were present in both populations (*right*) (**J**). Immunostained sections of day 20 vesicle-like structures revealed co-expression of CHX10 and Ki67 (**K**). By day 50, many vesicle-like structures lost their distinctive morphology and developed internal rosettes (**L**), but remained CHX10+/Ki67+ (**M**).

Fig. 2. Optic vesicle-like structures produce major neuroretinal cell types in a developmentally appropriate sequence

qPCR analysis of isolated OV-like structures was performed every 10 days from day 20–120 to follow expression of genes indicative of selected neuroretinal cell types (data shown are relative to day 20 for each gene) (A). ICC was used to corroborate neuroretinal cell type identity at day 80: retinal ganglion cells (B), amacrine/horizontal cells (C), bipolar cells (D), and photoreceptors (E–H).

Fig. 3. Early forebrain spheres produce multiple neural cell types

After 20 days of differentiation, EFB spheres expressed β III-TUBULIN (A–C), PAX6 (A), SOX1 (B), and OTX2 (C). After 70 days, nearly all cells expressed markers of mature neurons or glia, including GABA, β III-TUBULIN (D), tyrosine hydroxylase (TH), MAP2 (E), and GFAP (F). PCR analysis (G) at days 20 and 70 confirmed changes in transcription factor expression over time.

Fig. 4. Photoreceptor-like cells from optic vesicle-like structures display a characteristic electrophysiological signature

Cells undergoing electrophysiological analysis were loaded with sulphorhodamine (A) and later immunostained to confirm photoreceptor marker expression (B). Expression of multiple genes involved in phototransduction was determined at day 80 of differentiation (C). (D) Average current density measured from 15 photoreceptor-like cells (*solid circles*) and 3 non-photoreceptor cells (*open squares*) plotted against applied step voltage. (E) Ionic current responses measured at +40 and +20 mV from a representative voltage-clamped cell before (control) and after (+TEA) application of TEA. TEA sensitive outward currents were calculated by mathematical subtraction. (F) I-V curve showing average steady state current amplitudes of the photoreceptor-like cells before (*solid circles*) and after (*solid squares*) bath application of TEA. (G) Time course of the effect of 8-br-cGMP on current amplitude in a representative photoreceptor-like cell. (H) Ionic current traces obtained in response to voltage pulses delivered before (control) and during (+8-br-cGMP) administration of 8-br-cGMP. The horizontal line represents zero current. (I) I-V curve showing average current responses before (*solid circles*) and during (*solid squares*) 8-br-cGMP administration. Results shown are an average of 4 experiments $\pm$ SEM.

Fig. 5. Optic vesicle-like structures can be directed to an RPE fate

RPE was rarely observed in isolated OV-like structures after 50 days of differentiation (A). With the addition of Activin A between day 20–40, a subset of these structures became pigmented (B), whereupon they could be manually isolated and cultured separately (C). Plated pigmented structures were grown in the presence of FGF2, EGF, and heparin to promote outgrowth of cells (D). Upon removal of mitogens, RPE adopted its typical appearance (E) and expressed characteristic markers (F). Activin A-treated OV-like structures expressed higher levels of RPE-associated genes (G) and lower levels of neuroretinal-associated genes (H) by qPCR. Monolayers of RPE (I) were loaded with Fura-2 AM and stimulated with ATP while being monitored via epifluorescence imaging (J) to record changes in $[Ca^{2+}]_i$ over time (K). Panel J is an epifluorescence image of the cells shown in panel I. Cells identified in panel J were used to generate the tracing in panel K. The bar in panel K shows the timing of ATP administration.

Fig. 6. Human iPSC lines produce optic vesicle-like structures indistinguishable from hESCs
 Variability was observed by qPCR in the expression of anterior neural/eye field genes at day 10 of differentiation across multiple hESC and hiPSC lines (A–B and Supporting Information Fig. S4), with low-expressing lines adopting a non-neural, epithelial-like identity (C–D). Higher relative expression levels of *DKK1* (E) and, to a lesser extent, *NOGGIN* (F), at day 2 correlated with higher relative expression levels of anterior neural/eye field genes at day 10. OV-like structures generated from hiPSCs (G) uniformly expressed CHX10 (H) after 20–25 days. Acquisition of an anterior neural/eyefield fate at day 10 was enhanced through the addition of DKK1 and NOGGIN from day 2–4, as determined by qPCR (data expressed relative to untreated cultures) (I). DKK1- and NOGGIN-treated hiPSC cultures yielded colonies at day 10 with neuroepithelial morphology (J) that expressed anterior neural/eye field markers (K). FACS analysis demonstrated increased expression of PAX6 (L) and CHX10 (M) after 10 and 20 days of differentiation, respectively, in DKK1- and NOGGIN-treated hiPSC cultures.

Fig. 7. Differentiation and testing of hiPSCs derived from a patient with gyrate atrophy Fibroblasts (A) from a patient with gyrate atrophy (GA) due to an A226V mutation in OAT were reprogrammed to hiPSCs (B). (A226)OAT hiPSCs formed neural rosettes (C) at day 10 of differentiation that expressed anterior neural/eye field markers (D). Further maturation yielded multiple retinal cell types, including photoreceptor-like cells (E) and RPE (F). OAT activity in RPE derived from (A226)OAT hiPSCs was significantly less than that of control RPE from human prenatal eyes, hESCs, and hiPSCs (G). OAT enzyme activity in (A226)OAT hiPSC-RPE was restored in the presence of 600 μM vitamin B₆ (H), or following gene repair via BAC-mediated homologous recombination (I). **p<0.0005; ***p<0.0001. Panels G–I are representative examples of 3 independent experiments.