Treatment Paradigms for Retinal and Macular Diseases Using 3-D Retina Cultures Derived From Human Reporter Pluripotent Stem Cell Lines

Rossukon Kaewkhaw,1,2 Manju Swaroop,3 Kohei Homma,*,1 Jutaro Nakamura,†,1 Matthew Brooks,1 Koray Dogan Kaya,1 Vijender Chaitankar,1 Sam Michael,3 Gregory Tawa,3 Jizhong Zou,4 Mahendra Rao,5 Wei Zheng,3 Tiziana Cogliati,1 and Anand Swaroop 1

1Neurobiology-Neurodegeneration & Repair Laboratory, National Eye Institute, National Institutes of Health, Bethesda, Maryland, United States
2Research Center, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand
3National Therapeutics for Rare and Neglected Diseases, National Center for Advancing Translational Sciences, National Institutes of Health, Rockville, Maryland, United States
4iPSC Core, Center for Molecular Medicine, National Heart, Lung, and Blood Institute, Bethesda, Maryland, United States
5The New York Stem Cell Foundation Research Institute, New York, New York, United States

Correspondence: Anand Swaroop, Neurobiology-Neurodegeneration & Repair Laboratory, National Eye Institute, National Institutes of Health, Bethesda, MD 20892, USA; swaroopa@nei.nih.gov.

Current affiliation: *Department of Physiology, Nippon Medical School, Tokyo, Japan.
†Department of Ophthalmology, Yokohama City University School of Medicine, Yokohama, Japan.

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We discuss the use of pluripotent stem cell lines carrying fluorescent reporters driven by retinal promoters to derive three-dimensional (3-D) retina in culture and how this system can be exploited for elucidating human retinal biology, creating disease models in a dish, and designing targeted drug screens for retinal and macular degeneration. Furthermore, we realize that stem cell investigations are labor-intensive and require extensive resources. To expedite scientific discovery by sharing of resources and to avoid duplication of efforts, we propose the formation of a Retinal Stem Cell Consortium. In the field of vision, such collaborative approaches have been enormously successful in elucidating genetic susceptibility associated with age-related macular degeneration.

Keywords: human retina development, disease modeling, photoreceptor, high throughput screening, chemical screens, drug discovery, transcriptome, next generation sequencing, three-dimensional organoid culture, fluorescent reporter

Retinal and macular degeneration are a major cause of incurable blindness worldwide. Mutations or variations in almost 250 genes have been associated with retinal diseases (www.sph.uth.tmc.edu/Retnet/, in the public domain); in a majority of these, the dysfunction or death of photoreceptors leads to vision loss.1 Photoreceptors are specialized neurons that capture and transmit visual information and have high metabolic demand for functional maintenance. Gene replacement strategies have been successful, at least to some extent, for treatment of Leber congenital amaurosis (LCA) caused by RPE65 mutations2,3 and are being attempted for several inherited retinal degenerations.4 Preclinical studies in animal models show promise for further gene therapy applications, for example, for retinal degeneration caused by RPGR mutations.5,6 Clinical trials are also in progress to evaluate the safety (and possible efficacy) of transplanting pluripotent stem cell-derived retinal pigment epithelium (RPE) in patients with macular degeneration.7 Neuroprotection is another viable approach for augmenting photoreceptor survival.8 Genome- and network-based drug design, though in infancy, should be a useful conduit for personalized medicine.9,10

Pioneering studies have suggested the feasibility of restoring visual function by transplanting fetal retina or green fluorescence protein (GFP)-tagged immature photoreceptors.11,12 Much of our understanding of photoreceptor development is based on studies in mice and zebrafish.13–15 Derivation of photoreceptors from human pluripotent stem cells (PSCs) has now permitted investigations of developmental and pathogenic mechanisms.16–19 Self-organizing three-dimensional (3-D) neural retina (NR), generated in a culture dish from human embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs),20–22 now provides exciting opportunities for exploring gene regulatory networks underlying development, creating disease models, and designing new treatments.23–28 We recently reported studies on human photoreceptor development using the H9 human (h)ESC line carrying GFP reporter...
under control of the promoter of cone-rod homeobox (CRX) gene that regulates differentiation of both rod and cone photoreceptors. Fluorescent reporters are convenient markers for lineage- and developmental stage–specific identification of molecules and/or cell types within a tissue. Here, we discuss the generation and use of reporter PSCs for elucidating human retinal differentiation and disease pathogenesis and for developing novel treatment paradigms.

**HUMAN RETINAL DEVELOPMENT IN 3-D CULTURE**

During embryonic development, retinal organogenesis initiates with the emergence from the eye field of the optic vesicle (OV), a neuroepithelium capable of producing neural retina (NR) and RPE upon invagination of the optic cup (Fig. 1A). The retinal neuroepithelium includes distinct pools of multipotent progenitor cells, giving rise to multiple retinal cell types. One glial and six major neuronal cell types originate in stereotypical order from retinal progenitors in a sequence of events that are coordinated by extrinsic and intrinsic factors. With development proceeding in a central to peripheral order, retinal ganglion cells (RGCs) differentiate first, followed by cone photoreceptors, horizontal and amacrine neurons, and finally rod photoreceptors and bipolar neurons conclude neurogenesis before differentiation of Müller glia. Pluripotent stem cells can be differentiated in 3-D culture to produce retinal organoids, providing probably the closest approximation to the developing human retina (Fig. 1B). Early in the differentiation process, aggregates from PSCs cultured in defined differentiation media spontaneously express site-specific markers characteristic of eye field (e.g., RAX, SIX3, and PAX6). Structures resembling the OV and evaginating from 3-D organoids are composed of cells expressing characteristic retinal progenitor markers, such as RAX, VSX2, and PAX6. Within these vesicles, retinal cell types are born in a conserved order similar to that in the developing retinal neuroepithelium, expressing characteristic cell type–specific markers, and eventually stratifying in distinct neuronal layers. In addition, histologic appearance and organization in retinal layers in 3-D cultures are comparable to that in the developing retina, with photoreceptors positioned apically and ganglion cells located at the basal side of the NR (Fig. 1B). Although a rare event, human photoreceptors in 3-D retinal cultures can produce outer segments, a hallmark of functional differentiation. Retinal progenitors and photoreceptors derived from human PSCs have the ability to differentiate and may even integrate upon transplantation in the mouse retina. Thus, 3-D retinal “organoids” can provide a powerful in vitro model to investigate intrinsic transcriptional networks and extrinsic signaling components underlying human retinal morphogenesis.
FLUORESCENT REPORTER GENES TO TARGET DIFFERENTIATION OF RETINAL NEURONS

Birth, differentiation, and stratification of neurons in the vertebrate retina follow an evolutionarily conserved order that is reflected by expression of specific sets of genes. Thus, fluorescent reporters controlled by promoters of such genes can help identify defined developmental stage(s) or individual cell type(s) (Table 1). Specific promoter-reporter constructs can be inserted at unique, presumably neutral, sites in the human genome (such as AAVS1 on chromosome 19, CLYBL on chromosome 13) by zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), or CRISPR/Cas9. 36–38 We have generated four human reporter constructs, using NRL, RAX, CRX, and OTX2 promoters, for insertion at the AAVS1 site in hESCs and hiPSCs (Fig. 2). These constructs have been tested by electroporation in neonatal mouse retina, as described by Kaewkhaw et al. 39 Another approach for targeting reporters to specific sites is by knock-in using homologous recombination (Fig. 3). 40,41 While labor-intensive, the knock-in strategy does not require prior characterization of the promoter and offers expression of the reporter in native chromatin context, thereby more faithfully reflecting the endogenous gene expression pattern.

| Gene ID | Initial Expression Day in Culture | Reference |
|---------|-----------------------------------|-----------|
| NRL/C0  | Day 70–80                          | 45        |
| RAX/C0  | Day 10–15                          | 67        |
| CRX/C0  | Day 20–30                          | 29        |
| OTX2 enhancer/C0 | Day 10–20 | 68        |
| OTX2 promoter/C0 | Day 10-20 |            |

TABLE 1. Selected Human Retinal Promoters/Enhancers Used by Our Group for Driving Reporter Gene Expression In Vitro

**FIGURE 2.** Donor vectors for insertion of fluorescent reporters at the AAVS1 site using zinc finger nucleases. The use of different color spectra can allow the concomitant detection of more than one reporter.
GENERATION OF 3-D RETINA FROM HUMAN PLURIPOTENT STEM CELLS EXPRESSING DEVELOPMENTALLY REGULATED FLUORESCENT REPORTERS

The 3-D retina protocol we use involves induction of OVs from floating aggregates (Fig. 4A) as described previously. In other instances, adherence of early-stage aggregates or confluent culture of PSCs can initiate the formation of retinal neuroepithelium in OV-like structures. Given the varied culture and differentiation conditions currently employed across laboratories, development progression and birthdates of retinal cell types in “real” time in vitro may differ among protocols. Thus, results/data comparison may be better achieved using “pseudo” times, defined as the time of appearance of a certain cell type or attainment of a defined developmental stage based on one or more molecular markers. The use of retina-specific promoter-driven fluorescent reporters stably transfected in PSCs facilitates the establishment of pseudo times for 3-D differentiating retina. For instance, in our in vitro differentiation system, photoreceptors are born...
between day (d)30 and d37, based on expression of GFP driven by the CRX promoter (Fig. 4B; d90 retina is shown).39 Similarly, progression of differentiation in 3-D retina could be monitored using combinations of fluorescent reporters (see Fig. 2). Photoreceptor and other retinal cell types tagged with cell- and time-specific fluorescent markers can also be isolated by flow sorting (Fig. 4C) for molecular studies, including gene expression, regulation, and network analysis (Fig. 4D). Finally, fluorescent reporters can facilitate the selection of subpopulations of progenitors or postmitotic precursors testable in preclinical experiments as a proof of principle for the establishment of the cell of choice for transplantation.45–47

**INSIGHTS INTO HUMAN PHOTORECEPTOR DEVELOPMENT**

Next-generation sequencing has facilitated the identification of gene networks that control photoreceptor differentiation and/or disease pathology.9,48 We recently reported the transcriptome of developing photoreceptors (GFP+/CRX+ cells from d37 to d90) in 3-D human retina cultures derived from a CRXp-GFP hES reporter cell line.39 Our analysis identified cell type- and developmental stage-specific gene networks and cell surface markers that can be exploited for cell replacement and other treatment strategies. Further differentiation of 3-D retina culture leads to maturation of late-stage neurons, including rod photoreceptors. We have now performed transcriptome profiling of GFP+/CRX+ photoreceptors at d134 and d220 and demonstrated predicted patterns of cone and rod maturation, as revealed by expression of transcription regulatory proteins and phototransduction genes (Fig. 5). The expression of several early eye/retina field transcription factors and signaling proteins, including PAX6, SIX6, VSX2, LHX2, NOTCH1, ONECUT1, ONECUT2, and NEUROG2, is downregulated at d134 (Fig. 5A). PAX6 and NEUROG2 are undetectable by d220. Almost all genes associated with phototransduction, with the exception of OPN1LW and GUCY2F, are expressed by...
From the transcriptome profiles of GFP+/CRX+ human photoreceptors (from H9 hES cells) we extracted a panel of genes encoding cell surface molecules (Fig. 5C) and compared them with that of mouse rod- (from Nrpl-GFP/wild-type retina) and cone-like cells (from Nrpl-GFP/Nrpr−/− retina),35,49 revealing concordant candidate surface markers. We have reported several human cone cell surface proteins, such as RTN4R1L, ST3GAL5, GNMT2, and EPHA10, and cell surface markers of human rod photoreceptors, including GABRR2 and CNGB1, from early developmental stages.39 By specifically extracting CD molecules between d37 and d220, we observed that the expression of CD130, CD26, and CD44 increased with differentiation, with high transcript levels at d134 and d220 (late-stage markers) (Fig. 5C). In contrast, CD29 represented an early-stage marker as its expression was high at d37 and d47 and was subsequently downregulated. We suggest that a combination of CD29 and CD166 could selectively purify early-stage photoreceptors (Fig. 5C). CD230 and CD119 appear to be good candidate differentiation markers for human photoreceptors. Notably, genes of the solute carrier (SLC) superfamily encoding membrane-bound transporters (e.g., SLC40A1, SCL23A2, and SLC17A5) are solute carrier proteins transporters (e.g., SLC40A1, SCL23A2, and SLC17A5) are solute carrier proteins that can be exploited to develop chemical compounds that modulate photoreceptor physiology. Cell surface molecules have been previously identified for purifying developing mouse photoreceptors for transplantation.46,47 However, the pattern of expression of mouse cell surface markers is not fully reproduced in differentiating human retina in vivo and in vitro. While we detect CD47 and CD133 in flow-sorted human photoreceptors, CD24 is absent from hESC-derived photoreceptors.39 We also identified KCNQ3, which has been proposed together with Cacna2d4 and Cnga1 as a candidate for the enrichment of mouse rod photoreceptors.39 39 Our comprehensive gene expression profile of hESC-derived developing photoreceptors provides a reference catalog of candidate cell surface molecules that can be exploited to isolate human cone and rod photoreceptors for gene network analysis and cell replacement therapy.

### HIGH-TROUGHPUT SMALL-MOLECULE SCREENING USING FLUORESCENT REPORTER LINES

High-throughput screening (HTS) of chemical libraries has become a critical tool in basic biology and drug discovery. Combining HTS with human PSCs and derived cells offers a valuable strategy to identify compounds that modulate development or affect specific cell lineages. The 3-D retinal development model we have established allows us to identify and segregate multiple cell types in overlapping developmental stages over a long time frame of 200+ days in culture, permitting the use of distinct HTS assays with the cells at specified periods in culture or with sorted specific cell types. For example, HTS of chemical libraries can be used to identify compounds that facilitate retinal progenitors to produce a homogeneous population of cone or rod photoreceptors. With the approved drug collection and bioactive compound collection, HTS of patient iPSC-derived cells can be used for rapid drug repositioning and for identifying new drug targets. We can also perform HTS of patient iPSC-derived retinal cells for discovering therapeutic compounds that can rescue a specific molecular or cellular phenotype, or use HTS as a “chemical genetic approach” for dissecting molecular regulatory networks underlying normal or abnormal development of photoreceptors or other retinal cells.

Our strategy of HTS using 3-D retinal culture from the CRXp-GFP H9 hESC line is illustrated in Figure 7. Our initial goal has been to develop a prototype screening system as a demonstration to identify compounds that accelerate photoreceptor differentiation by stimulating expression of CRX and, consequently, its downstream targets. Here we describe our HTS strategy in some detail, and highlight the importance of appropriate controls and establishing reproducibility that are critical for success.

To generate a large number of cells, a requirement for HTS, we used suspension culture of aggregates. Almost 10^3 cells/aggregate at d0. Aggregates were produced within 20 days when starting with 9 x 10^3 cells/aggregate at d0. Aggregates were then
dissociated into single cells, a relatively easy step at this early stage, taking generally 15 minutes with Accumax (containing DNase; Innovative Cell Technologies, San Diego, CA, USA). Optimum plating density was crucial to obtain reliable and interpretable image results. To maintain postseeding cell survival of PSCs and their derivatives, we selectively inhibited Rho-associated, coiled coil-containing protein kinase (ROCK). In our studies, use of ROCK inhibitor Y-27632 significantly improved cell survival after 24-hour treatment, and fewer cells were needed for primary assay screening (reduced from $8 \times 10^3$ to $4 \times 10^3$ cells per well in 1536-well plate format). Expression of CRXp-GFP reporter in differentiating hESCs provided the reading output for determining whether a compound accelerates photoreceptor differentiation as described below.

**Optimization of Cell-Based GFP Assay**

To develop a sensitive and reproducible assay for HTS, we first optimized the cell seeding density in 384- and 1536-well plates using GFP-positive embryoid bodies (EBs) at d34 and d51. Five EBs per well were dispensed in a 24-well plate and rinsed one time with PBS. Embryoid bodies were dissociated in 1 mL Accumax (Innovative Cell Technologies) at 37°C for 10 minutes and triturated 10 times, followed by 15-minute incubation. The EBs were triturated once again, and dissociated cells were passed through a 40-μm filter. Cells were pooled, centrifuged at 180 $\times$ g for 3 minutes, and resuspended in 0.5 mL Dulbecco's modified Eagles's medium (DMEM) + retinoic acid (RA) + N2 + ROCK inhibitor. Cells were counted and seeded in either 384- or 1536-well plates. We obtained a strong signal with good discrimination between the GFP d51 positive control and GFP d34 with $1.6 \times 10^4$ cells per well in the 384-well plates and $5 \times 10^3$ cells per well in the 1536-well plates.

We chose to use three different small-molecule libraries. A library of pharmacologically active compounds (LOPAC) containing 1280 compounds was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). The library of US Food and Drug Administration (FDA)-approved drugs and MIPE [Mechanism Interrogation PlatE] collection_1912 compounds (approved for clinical trials) were set up internally. Compounds were dissolved in dimethyl sulfoxide (DMSO) as 10-mM stock solutions and were further diluted in 384-well plates to seven concentrations at a 1:5 ratio. A CyBi-Well dispensing station with a 384-well head (Cybio, Inc.)

**FIGURE 6.** In vivo differentiation of CRXp-GFP H9 hESC-derived retinal cells from 3-D cultures. CRX+/GFP+ cultures at d58 were dissociated, and cells were injected in the subretinal space of recipient adult C57Bl6 mice. Eyes were collected 4, 6, and 14 weeks post injection, resulting in combined differentiation times of 12 weeks (w), 14w, and 22w, respectively. (A–E) Most transplanted CRX+/GFP+ cells formed polarized “rosette” structures with the apical side facing inward based on phalloidin staining (arrows) (A). By 22w, a majority of rosettes were labeled with rhodopsin (4D2) antibody in their apical center (arrowbeads) (B, C). (D) Occasional, faint S opsin staining could be observed as early as 14w on the apex of CRX+/GFP+ photoreceptor cells (asterisk). (E) A small number of CRX+/GFP+ photoreceptors expressed L/M opsin at 22w (arrows). INL, host inner nuclear layer; ONL, host outer nuclear layer; SRS, host subretinal space; a, apical; b, basal. Scale bars: 20 μm.
Woburn, MA, USA) was used to reformat diluted compounds in 384-well plates to 1536-well plates. It was important for our efforts that screening could be performed in 384- or 1536-well plates to reduce costs.

Having optimized cell plating and GFP readout, and having established the usability of 384- and 1536-well formats at different stages of compound screening, we performed an initial assay (Table 2) as described below. In brief, \(5 \times 10^3\) cells/well were plated using the Multidrop Combi Dispenser (Thermo Scientific, Waltham, MA, USA) and cultured for 48 hours in 5 \(\mu\)L growth medium containing ROCK inhibitor. The compound libraries were transferred in a volume of 23 nL per well using the NX-TR Pintool (Wako Scientific Solutions, San Diego, CA, USA). The cells were cultured with compounds for 24 hours at 37\(^\circ\)C. The primary screen was conducted in six plates with serial compound dilution: one DMSO control plus five concentrations (0.07, 0.37, 1.84, 9.2, 46 \(\mu\)M) of compounds. In each plate, a column of \([d51-GFP]\) cells was used as a positive control, and DMSO-treated cells served as a negative control. In order to reduce nonspecific fluorescence signals generated by serum proteins and cell surface proteins, a quencher dye (1 \(\mu\)L/well) was added 24 hours post compound treatment with a Multidrop-Combi dispenser. We observed that DMSO-treated cells remained viable and healthy in 1536-well plates for at least 48 hours as assessed by their morphology and ability to remain adherent using cell imaging.

From the primary screening results, we selected active compounds exhibiting low AC_{50} (concentration required to elicit a 50% response in an in vitro assay) and high efficacy for further confirmation. The hit confirmation assay was performed using the same CRXp-GFP assay with an 11-point serial dilution at 1:3 ratio of each selected compound in duplicate in parallel with a compound cytotoxicity assay (Fig. 8). After confirmation of primary screening hits, we carried out the following tertiary assay to further confirm the compound activities. Global gene expression analysis is an excellent starting tool to define signaling pathways or gene regulatory networks stimulated by compounds augmenting CRX expression. To avoid irrelevant data, only GFP\(^+\) cells following compound treatment would be purified for analyses.

**Informatics Analysis**

The 100% signal was defined from the control wells devoid of compounds, and the basal signal was obtained from the wells of cells treated with DMSO solution. Data from the primary screen were analyzed using customized software that performs normalization and corrects for screening artifacts using a combination of control wells within and between the screening plates. The signal-to-background ratio was calculated as a comparison between d51 GFP\(^+\) control and d34− cells treated with DMSO. We first ran quality control on the plate data by visual inspection, masking wells showing erroneous signals, for example, localized groupings of wells exhibiting enhanced or inhibited signal. We then performed intraplate normalization. We defined two control well types: a neutral control well, which contained DMSO and progenitor cells (basal), and a positive control well, which contained a known stem cell stimulator and progenitor cells (enhanced). The rest
of the wells contained test compounds and progenitor cells. The data were then normalized according to the following equation:

\[
\text{%Activity} = \frac{100 \times (\text{Test Compound Signal} - \text{Basal Signal})}{(\text{Control Signal} - \text{Basal Signal})}
\]

With this definition, test compounds with near basal signals will have 0% activity (inactive); test compounds with signals near that of the positive control will have 100% activity (enhancers); and test compounds with signals less than basal will have negative activity (inhibitors). This normalization controls for small differences in protocol implementation across plates, for example, slight differences in exposure time and incubation time. We also applied an intraplate background correction, subtracting the median well signal (after normalization) from all well signals. This correction accounts for changing patterns of background response over the course of the experiment. As a last step, we fit hill curves to the concentration-dependent intensities for each compound. From the curve fits, we determined the compound potency (AC50), and efficacy (% Activity max / % Activity min) and we chose compounds for confirmatory studies, with well-defined dose–response curves and exhibiting high potency and efficacy.

Overall our experiments showed that running a HTS screen with iPSC-derived human cells was feasible as long as careful attention was paid to establishing the assay. If the assay was miniaturized to 384- and 1536-well format, the overall cost was significantly reduced, with fewer cells needed and many fewer assay plates screened.

**FUTURE PERSPECTIVES**

These are exciting yet early days in stem cell biology and medicine. Here, we have presented a framework for using reporter PSC lines for investigating retinal biology, disease modeling, and development of novel treatment strategies. Fluorescent reporters facilitate targeted evaluation of a specific cell type or developmental stage for biological and clinical studies. We have generated several reporter constructs and are in the process of characterizing transfected hESCs. National Center for Advancing Translational Sciences (NCATS) has a large collection of over 400,000 compounds that can be utilized if and when HTS assays are designed for developmental studies or drug discovery to rescue phenotypes observed in patients’ iPSC derivatives. We may also involve the medicinal chemistry in NCATS for the lead optimization after identification and confirmation of active compounds from HTS. However, we realize that such investigations are labor-intensive and require extensive resources. To expedite scientific discovery by sharing of resources and to avoid duplication of efforts, we propose that interested scientists form a Retinal Stem Cell Consortium. In the field of vision, such large, collaborative approaches have been enormously successful in age-related macular degeneration genetics studies.\(^5\) \(^6\)

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