

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

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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* mutations^{2,3} 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



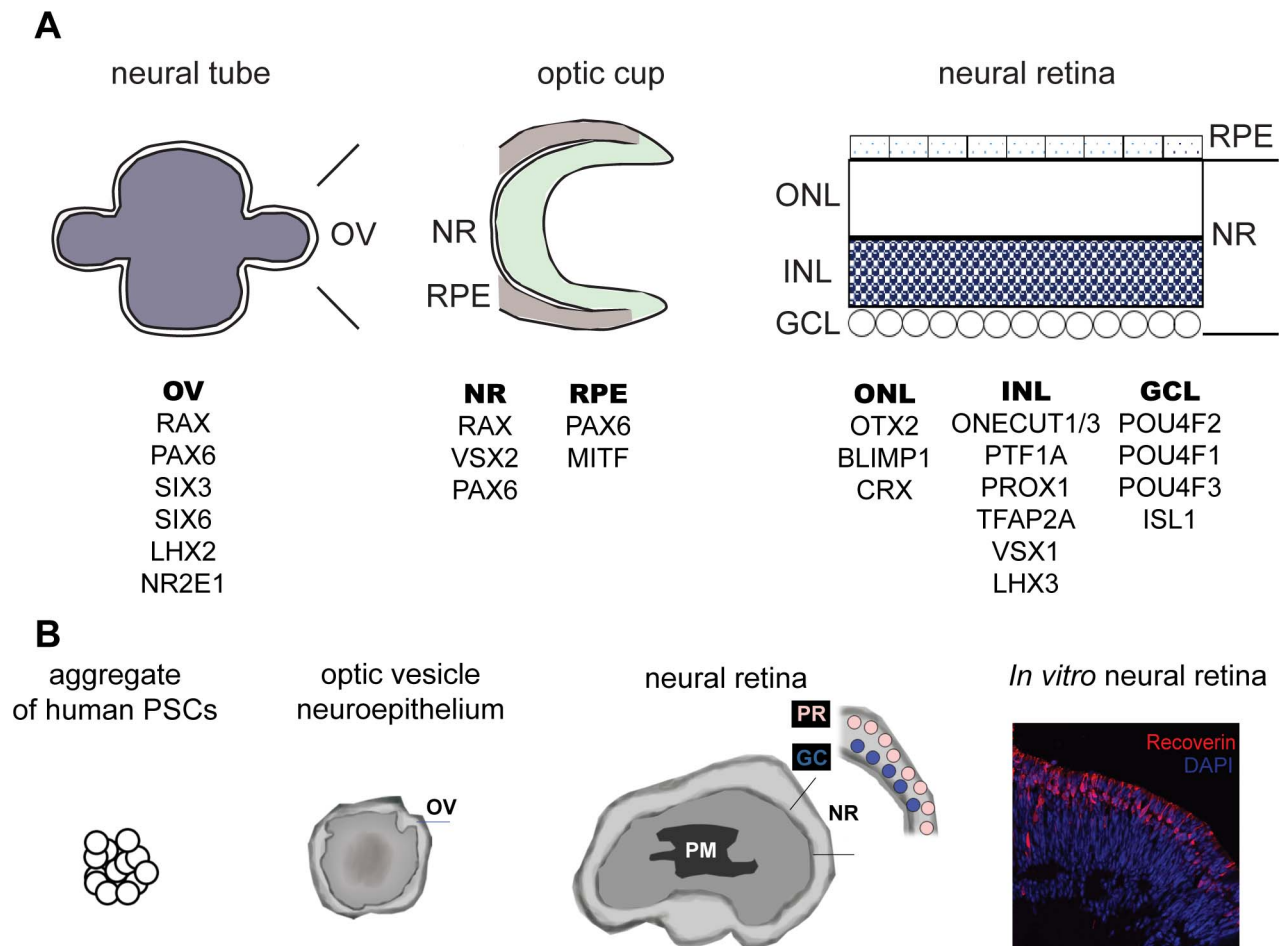


FIGURE 1. Development of neural retina and retinal pigment epithelium cells. (A) Neural retina (NR) and retinal pigment epithelium (RPE) cells develop from the optic vesicle (OV) that emerges from the neural tube. Invagination of the optic vesicle, placing the prospective NR alongside the prospective RPE, gives rise to the optic cup. Key transcription factors that regulate each developmental stage and determine the fate of cell types in the retinal layers are listed. (B) Three-dimensional retina *in vitro* can be generated by floating aggregates of PSCs, which form optic vesicles where NR and pigment mass (PM) develop. ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer; PR, photoreceptor; GC, ganglion cell.

under control of the promoter of cone-rod homeobox (CRX) gene that regulates differentiation of both rod and cone photoreceptors.²⁹ 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.³⁰ 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.^{31,32} 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.³³ 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.^{20,21} 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,²¹ a hallmark of functional differentiation. Retinal progenitors and photoreceptors derived from human PSCs have the ability to differentiate³⁴ and may even integrate³⁵ 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.

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

Gene ID	Promoter/ Enhancer		Initial Expression Day in Culture	Promoter Reference
	Start	End		
<i>NRL</i>	-4.6 kb	+17 kb	Day 70-80	45
<i>RAX</i>	-3.5 kb	0 kb	Day 10-15	67
<i>CRX</i>	-2.0 kb	+1 kb	Day 20-30	29
<i>OTX2</i> , enhancer	-16.9 kb	-14.9 kb	Day 10-20	68
<i>OTX2</i> , promoter	-3.0 kb	0 kb		

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.

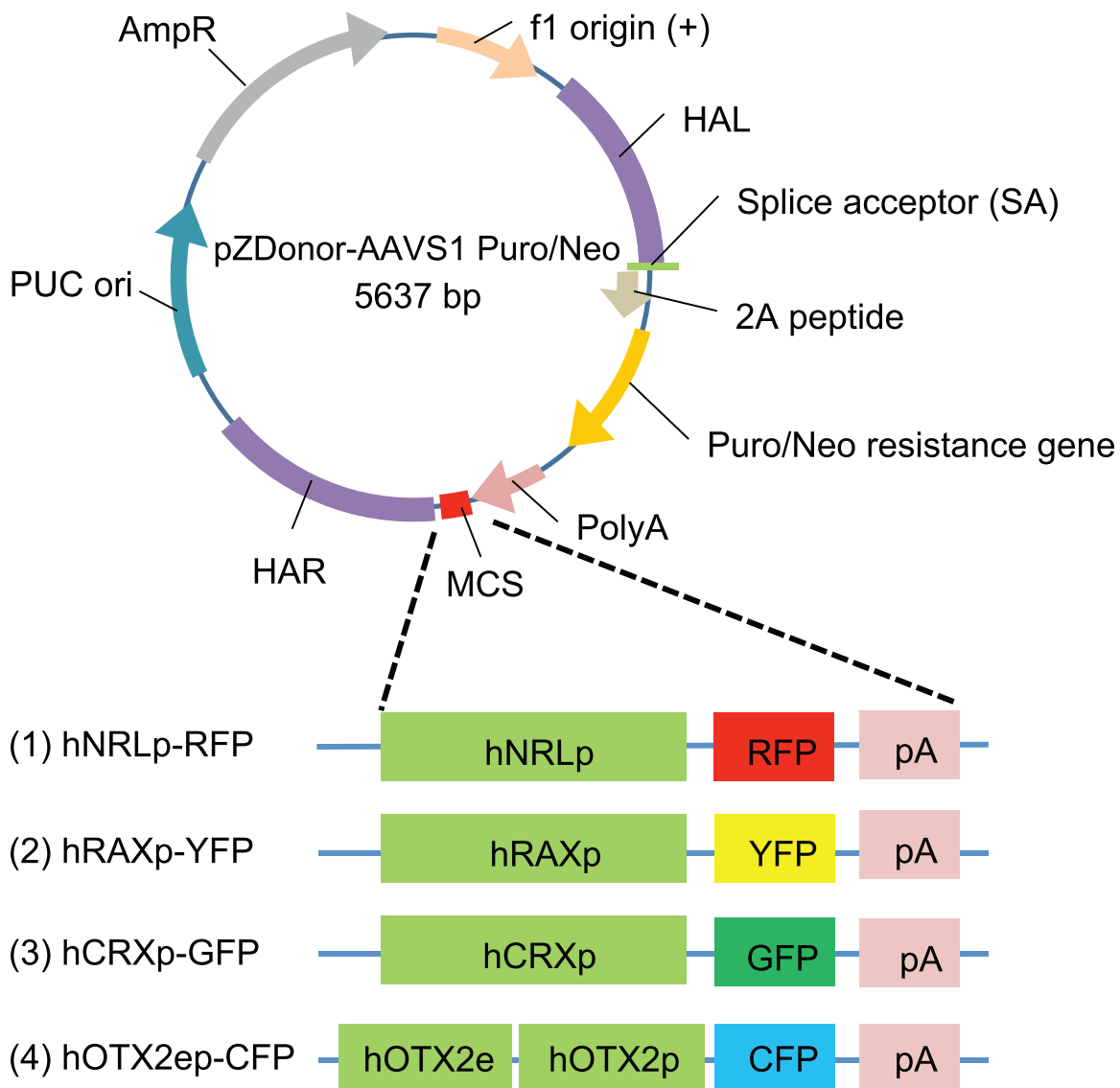


FIGURE 2. Donor vectors for insertion of fluorescent reporters at the AAVS1 site using zinc finger nucleases. The use of different color spectra can allow the concomitant detection of more than one reporter.

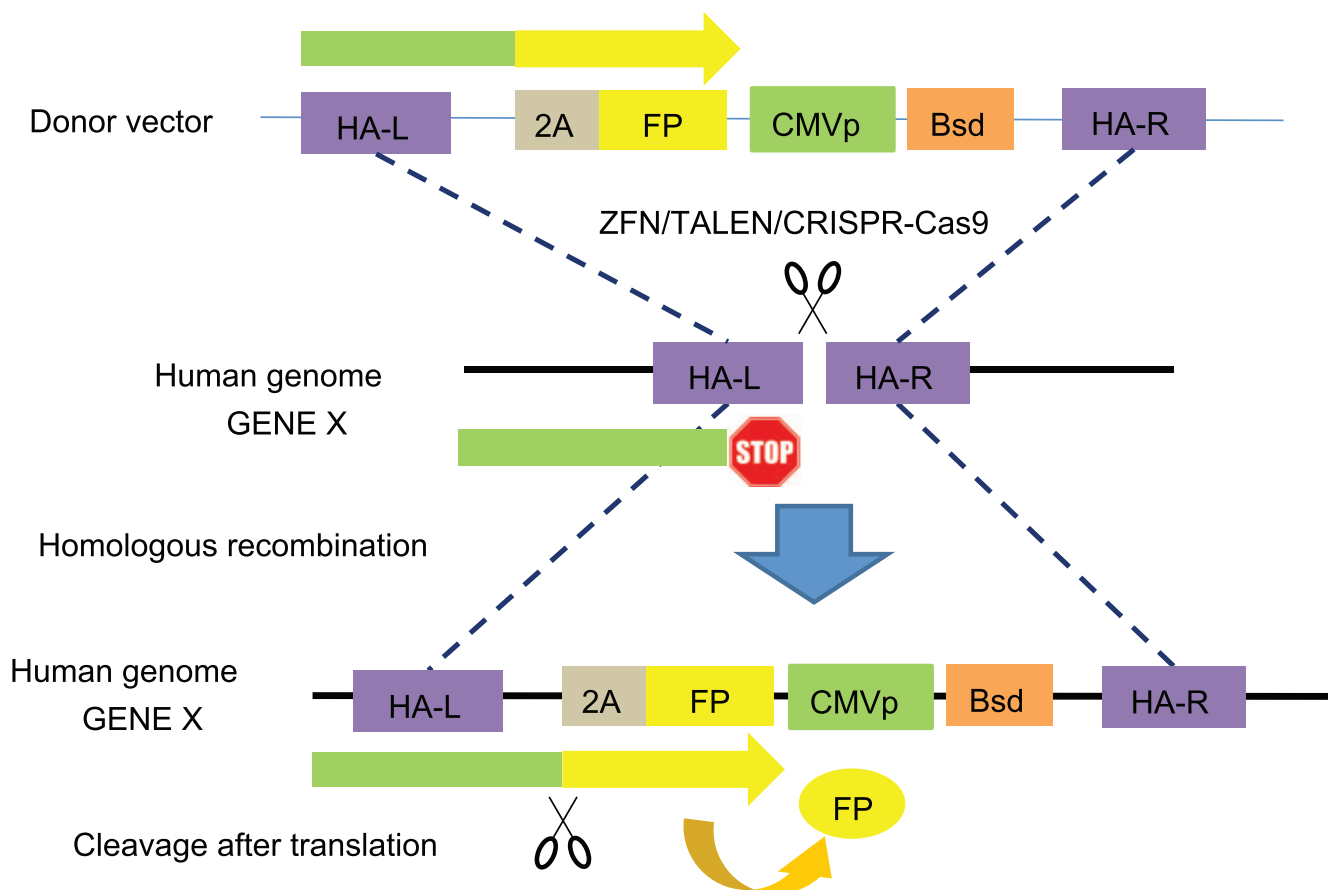


FIGURE 3. Strategy for knock-in using gene cleavage-induced homologous recombination. FP, fluorescent protein.

GENERATION OF 3-D RETINA FROM HUMAN PLURIPOTENT STEM CELLS EXPRESSING DEVELOPMENTALLY REGULATED FLUORESCENT REPORTERS

The 3-D retina protocol we use³⁹ involves induction of OVs from floating aggregates (Fig. 4A) as described previously.^{20,42} In other instances, adherence of early-stage aggregates or confluent culture of PSCs can initiate the formation of retinal neuroepithelium in OV-like structures.^{21,22,43} 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,⁴⁴ 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

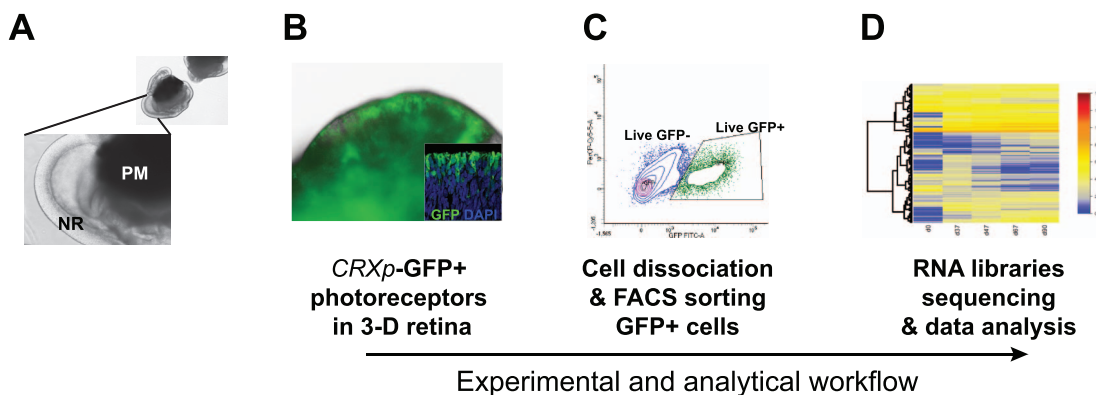


FIGURE 4. Three-dimensional retina derived from the *CRXp*-GFP H9 hESC reporter line. (A) Three-dimensional neural retina (NR) is generated from *CRXp*-GFP hESCs. (B) *CRXp*-GFP⁺ photoreceptors in NR can (C) be purified by fluorescence-activated cell sorting (FACS) for RNA isolation and next-generation sequencing. (D) Gene regulatory networks and cell surface molecules can be extracted from global gene expression analysis. PM, pigment mass.

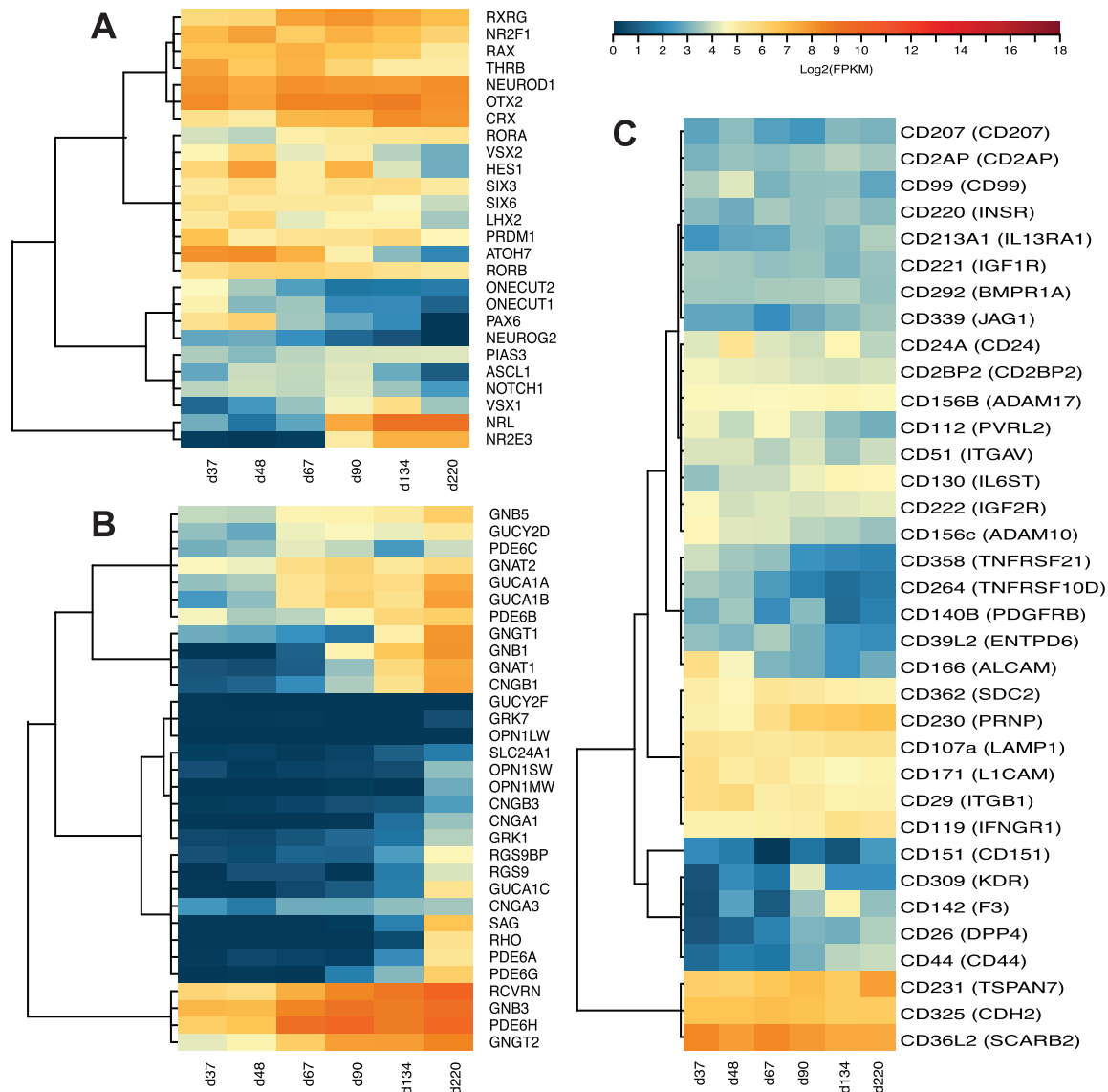


FIGURE 5. Expression profiles of selected genes in developing human photoreceptors in 3-D cultures. Clustering in the heat maps was performed using affinity propagation. The transcriptome data of d37, d47, d67, and d90 CRX+/GFP+ photoreceptors, shown here for comparison, has been reported recently.³⁹ (A) Transcription factors. (B) Phototransduction genes. (C) Cell differentiation (CD) markers (≥ 10 FPKM at d134 or d220). FPKM, fragments per kilobase of transcript per million mapped reads.

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 H9 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

d220 in 3-D retina (Fig. 5B). Our data indicate that downregulation of transcription factors involved in maintaining proliferation and progenitor cell multipotency is a prerequisite for the induction of genes associated with maturation of photoreceptors.¹³

From the transcriptome profiles of GFP+/CRX+ human photoreceptors (from H9 hESC cells) we extracted a panel of genes encoding cell surface molecules (Fig. 5C) and compared them with that of mouse rod- (from *Nrlp*-GFP/wild-type retina) and cone-like cells (from *Nrlp*-GFP/*Nrl*^{-/-}retina),^{45,49} revealing concordant candidate surface markers. We have reported several human cone cell surface proteins, such as RTN4RL1, ST3GAL5, GNGT2, 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 highly expressed in human photoreceptors at later stages (d134 and d220) (data not shown). Solute carrier proteins mediate transport of various molecules across the cell membrane,⁵⁰ and a better understanding of their function should assist in developing 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 *KCNV2*, which has been proposed together with *Cacna2d4* and *Cnga1* as a candidate for the enrichment of mouse rod photoreceptors.⁴⁶ 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.

TRANSPLANTATION AND IN VIVO DIFFERENTIATION OF HUMAN FLUORESCENT REPORTER-TAGGED RETINAL CELLS IN ANIMAL MODELS

Although feasible and highly promising,^{11,47,51} transplantation of photoreceptor precursors for tissue reconstruction still needs improvement in terms of efficiency of transplantation, cell survival, integration, and differentiation before potential cell replacement in humans. Much of the work has been conducted with allotransplantation of mouse photoreceptor precursors. Testing of human PSC derivatives in animal models has been of limited significance.^{35,52–55} The use of fluorescent reporter-tagged retinal cells derived from PSCs can facilitate optimization of such studies. We have transplanted retinal cells from *CRXp*-GFP H9 differentiating 3-D retina into wild-type mouse retina (Fig. 6). Though we did not observe much tissue integration, dissociated cells from 3-D retina could differentiate into GFP+ photoreceptors, which express opsin visual

pigments more rapidly compared with the rate of differentiation in culture.

DISEASE MODELING USING REPORTER iPSC LINES

Differentiated human cells with disease-specific phenotypes can be generated in a dish through iPSC technology,⁵⁶ complementing studies with model organisms that may not accurately replicate human biology. Neural retina and RPE have been generated through OV-like structures from iPSCs derived from patients with gyrate atrophy (OAT: A226V),⁴³ microphthalmia (VSX2: R200Q),⁵⁷ Best disease (BEST1: A146K, and N296H),⁵⁸ and retinitis pigmentosa.²⁴ Patient iPSC-derived RPE and NR can at times be distinguished by distinct phenotypes and physiological characteristics. Nonetheless, incorporation of appropriate reporter constructs into iPSC lines will help in identifying RPE and NR cell types at distinct stages of differentiation, thereby expediting molecular, structural, and functional analyses. For example, gene profiling of purified photoreceptors from heterogeneous 3-D retina derived from patients' iPSCs could reveal temporal changes in transcriptional regulation and/or pathways that contribute to disease pathology. We believe that the use of fluorescent reporters allows targeted evaluation of molecular and cellular abnormalities in patients' iPSC-derived retina, enabling high-throughput drug screens²⁵ and modeling of complex disease phenotypes.⁵⁹

HIGH-THROUGHPUT 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.^{60–62} Combining HTS with human PSCs and derived cells offers a valuable strategy to identify compounds that modulate development or affect specific cell lineages.⁶⁰ 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,⁶¹ 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 line³⁹ 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 5×10^5 cells/aggregate were produced within 20 days when starting with 9×10^3 cells/aggregate at d0. Aggregates were then

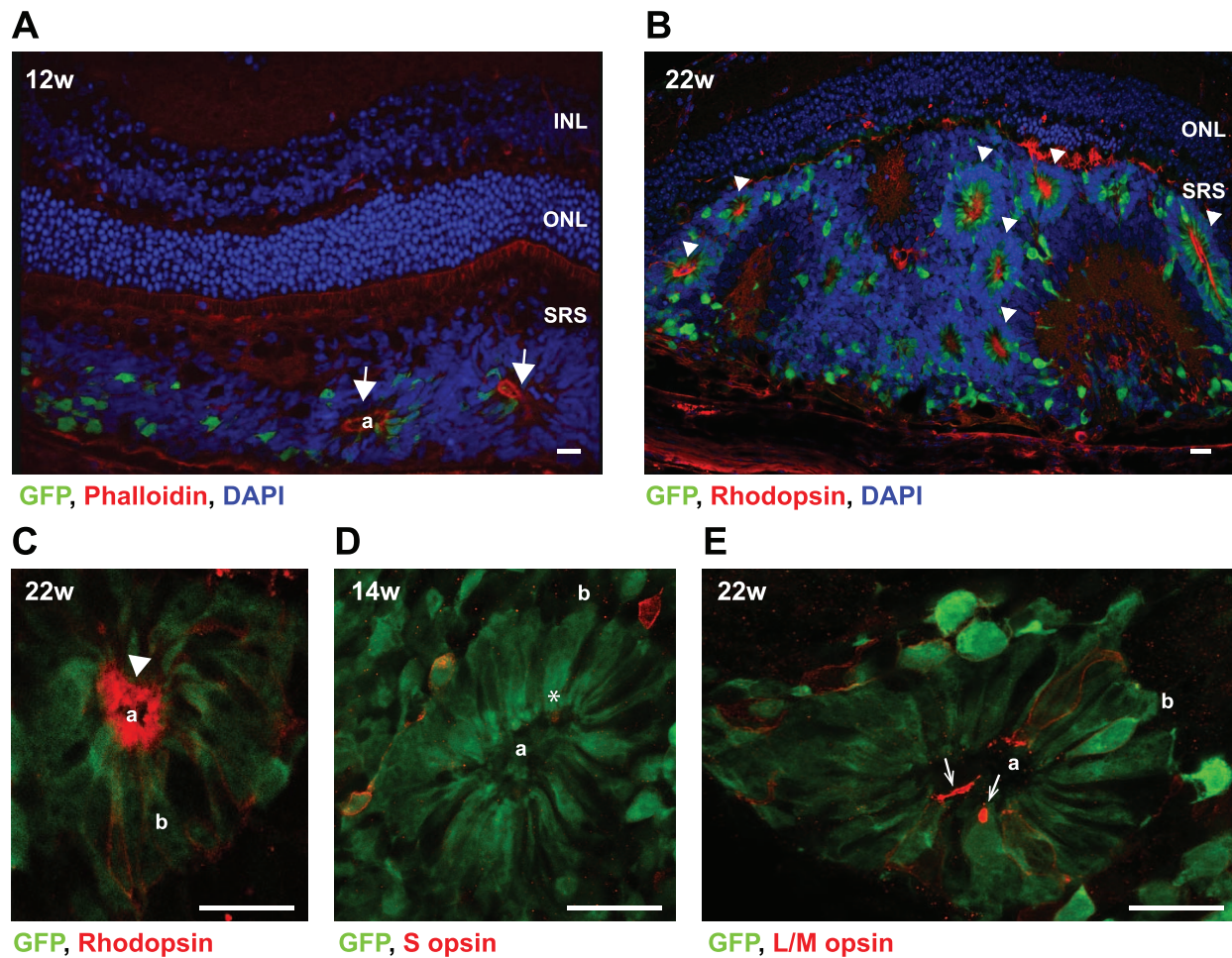


FIGURE 6. In vivo differentiation of *CRXp*-GFP H9 hESC-derived retinal cells from 3-D cultures. *CRX*+/*GFP*+ cultures at d58 were dissociated, and cells were injected in the subretinal space of recipient adult C57BL/6 mice. Eyes were collected 4, 6, and 14 weeks post injection, resulting in combined 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 (arrowheads) (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 μ m.

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).^{63,64} 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 8×10^3 to 4×10^3 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- μ m filter. Cells were pooled, centrifuged at 180g for 3 minutes, and resuspended in 0.5 mL Dulbecco’s modified Eagle’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 1.6×10^4 cells per well in the 384-well plates and 5×10^3 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_2800 compounds)-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.,

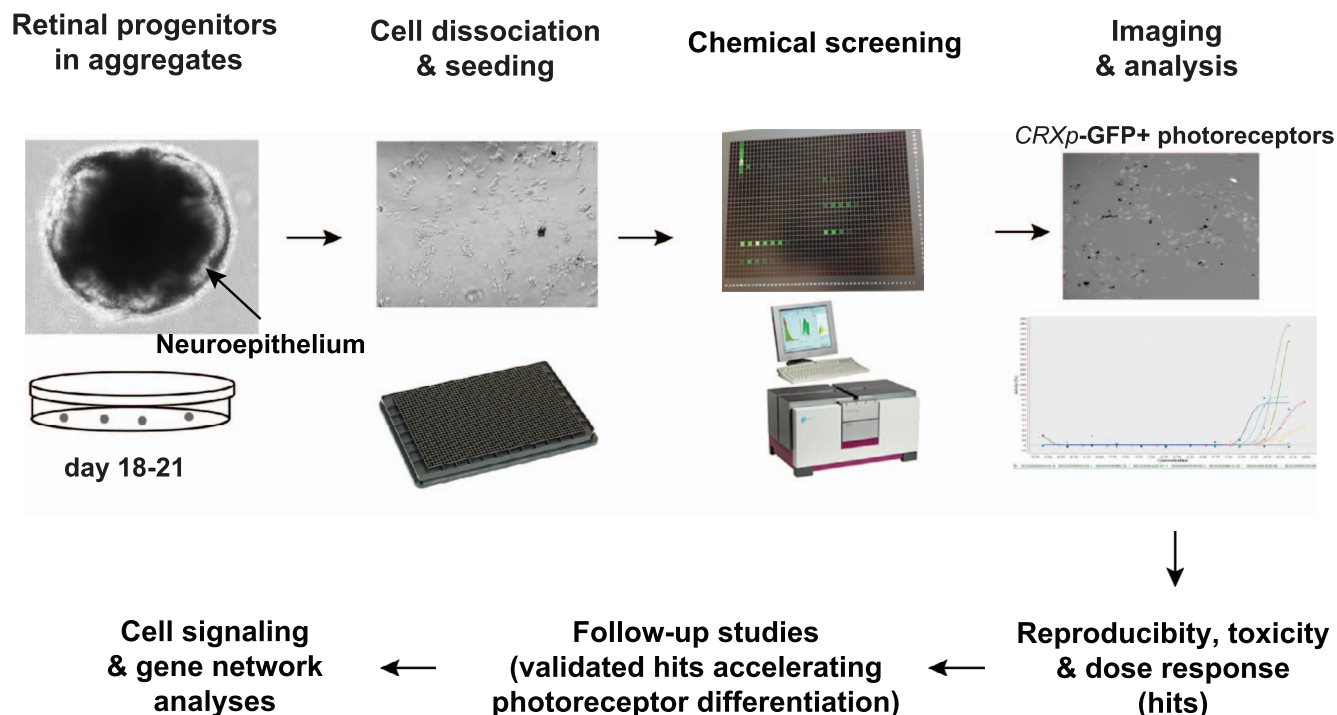


FIGURE 7. Schematic of HTS using 3-D retinal culture of the *CRXp-GFP* H9 hESC line.

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, 5×10^3 cells/well were plated using the Multidrop Combi Dispenser (Thermo Scientific, Waltham, MA, USA) and cultured for 48 hours in 5 μ 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°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 μ 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 μ 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

TABLE 2. Acumen-Based GFP Assay Protocol in 1536-Well Plate Format

Step	Parameter	Value	Description
1	Seed cells	5 μ L/well	5×10^3 cells/well; black, clear bottom
2	Centrifuge	1 min, 180g	Settle cell suspension in well
3	Incubate	2 d	37°C, 5% CO ₂
4	Compound	23 nL	Compound in DMSO
5	Incubate	24 h	37°C, 5% CO ₂
6	Quencher	0.5 μ L/well	Spin 30 s
7	Read	Acumen Cellista (TTP Labtech, Cambridge, MA, USA)	488 nm

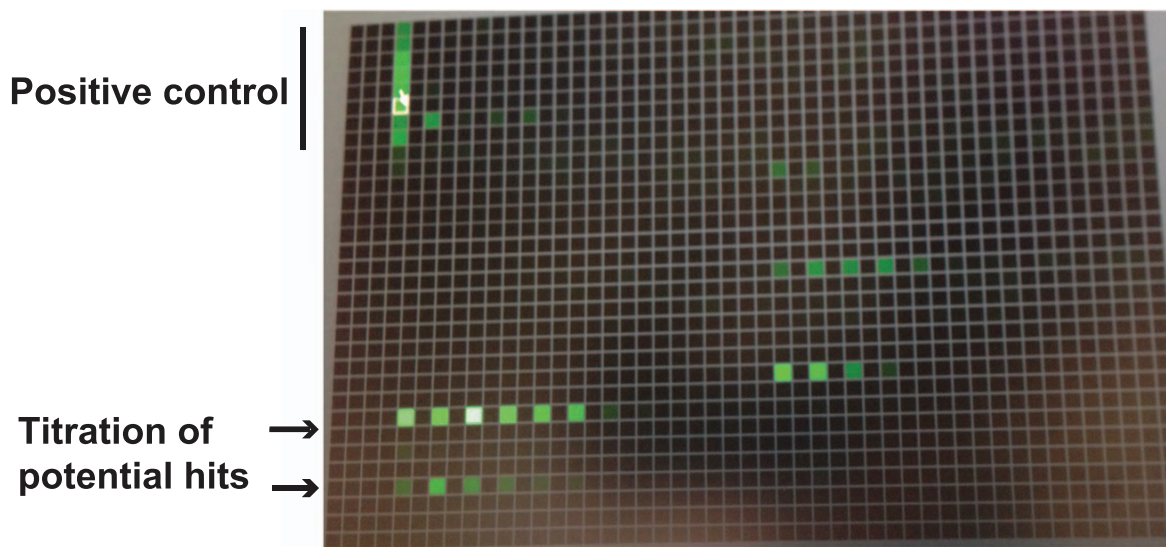


FIGURE 8. Plate view of small-molecule hit confirmation in 1536-well format. A total of 5600 compounds from four diverse molecular libraries were screened by HTS in a homogenous GFP cell-based assay. Validation of 32 primary actives using an 11-point dose-response titration was done at 3-fold serial dilutions of test compounds dissolved in DMSO at starting concentrations of 10 mM.

of the wells contained test compounds and progenitor cells. The data were then normalized according to the following equation:

$$\%Activity = 100 * \frac{(Test\ Compound\ Signal - Basal\ Signal)}{(Control\ Signal - Basal\ Signal)}$$

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 con-

structs 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.^{65,66}

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References

1. Wright AE, Chakarova CF, Abd El-Aziz MM, Bhattacharya SS. Photoreceptor degeneration: genetic and mechanistic dissection of a complex trait. *Nat Rev Genet.* 2010;11:273–284.
2. Jacobson SG, Cideciyan AV, Roman AJ, et al. Improvement and decline in vision with gene therapy in childhood blindness. *N Engl J Med.* 2015;372:1920–1926.

3. Bainbridge JW, Mehat MS, Sundaram V, et al. Long-term effect of gene therapy on Leber's congenital amaurosis. *N Engl J Med*. 2015;372:1887-1897.
4. Sahel JA, Roska B. Gene therapy for blindness. *Annu Rev Neurosci*. 2013;36:467-488.
5. Beltran WA, Cideciyan AV, Lewin AS, et al. Gene therapy rescues photoreceptor blindness in dogs and paves the way for treating human X-linked retinitis pigmentosa. *Proc Natl Acad Sci U S A*. 2012;109:2132-2137.
6. Wu Z, Hiriyan S, Qian H, et al. A long-term efficacy study of gene replacement therapy for RPGR-associated retinal degeneration. *Hum Mol Genet*. 2015;24:3956-3970.
7. Schwartz SD, Regillo CD, Lam BL, et al. Human embryonic stem cell-derived retinal pigment epithelium in patients with age-related macular degeneration and Stargardt's macular dystrophy: follow-up of two open-label phase 1/2 studies. *Lancet*. 2015;385:509-516.
8. Trifunovic D, Sahaboglu A, Kaur J, et al. Neuroprotective strategies for the treatment of inherited photoreceptor degeneration. *Curr Mol Med*. 2012;12:598-612.
9. Yang HJ, Ratnapriya R, Cogliati T, Kim JW, Swaroop A. Vision from next generation sequencing: multi-dimensional genome-wide analysis for producing gene regulatory networks underlying retinal development, aging and disease. *Prog Retin Eye Res*. 2015;46:1-30.
10. Chen Y, Palczewski K. Systems pharmacology links GPCRs with retinal degenerative disorders [published online ahead of print March 23, 2015]. *Annu Rev Pharmacol Toxicol*. doi:10.1146/annurev-pharmtox-010715-103033.
11. MacLaren RE, Pearson RA, MacNeil A, et al. Retinal repair by transplantation of photoreceptor precursors. *Nature*. 2006;444:203-207.
12. Seiler MJ, Aramant RB. Cell replacement and visual restoration by retinal sheet transplants. *Prog Retin Eye Res*. 2012;31:661-687.
13. Swaroop A, Kim D, Forrest D. Transcriptional regulation of photoreceptor development and homeostasis in the mammalian retina. *Nat Rev Neurosci*. 2010;11:563-576.
14. Ng L, Lu A, Swaroop A, Sharlin DS, Swaroop A, Forrest D. Two transcription factors can direct three photoreceptor outcomes from rod precursor cells in mouse retinal development. *J Neurosci*. 2011;31:11118-11125.
15. Stenkamp DL. The rod photoreceptor lineage of teleost fish. *Prog Retin Eye Res*. 2011;30:395-404.
16. Lamba DA, McUsic A, Hirata RK, Wang PR, Russell D, Reh TA. Generation, purification and transplantation of photoreceptors derived from human induced pluripotent stem cells. *PLoS One*. 2010;5:e8763.
17. 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.
18. 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.
19. Gamm DM, Wright LS. From embryonic stem cells to mature photoreceptors. *Nat Biotech*. 2013;31:712-713.
20. Nakano T, Ando S, Takata N, et al. Self-formation of optic cups and storable stratified neural retina from human ESCs. *Cell Stem Cell*. 2012;10:771-785.
21. Zhong X, Gutierrez C, Xue T, et al. Generation of three-dimensional retinal tissue with functional photoreceptors from human iPSCs. *Nat Commun*. 2014;5:4047.
22. Reichman S, Terray A, Slembrouck A, et al. From confluent human iPS cells to self-forming neural retina and retinal pigmented epithelium. *Proc Natl Acad Sci U S A*. 2014;111:8518-8523.
23. Boroah S, Phillips MJ, Bilican B, et al. Using human induced pluripotent stem cells to treat retinal disease. *Prog Retin Eye Res*. 2013;37:163-181.
24. Jin ZB, Okamoto S, Osakada F, et al. Modeling retinal degeneration using patient-specific induced pluripotent stem cells. *PLoS One*. 2011;6:e17084.
25. Grskovic M, Javaherian A, Strulovici B, Daley GQ. Induced pluripotent stem cells—opportunities for disease modelling and drug discovery. *Nat Rev Drug Discov*. 2011;10:915-929.
26. Ader M, Tanaka EM. Modeling human development in 3D culture. *Curr Opin Cell Biol*. 2014;31:23-28.
27. Tabar V, Studer L. Pluripotent stem cells in regenerative medicine: challenges and recent progress. *Nat Rev Genet*. 2014;15:82-92.
28. Lancaster MA, Knoblich JA. Organogenesis in a dish: modeling development and disease using organoid technologies. *Science*. 2014;345:1247125.
29. Furukawa A, Koike C, Lippincott P, Cepko CL, Furukawa T. The mouse Crx 5'-upstream transgene sequence directs cell-specific and developmentally regulated expression in retinal photoreceptor cells. *J Neurosci*. 2002;22:1640-1647.
30. Bermingham-McDonogh O, Reh TA. Regulated reprogramming in the regeneration of sensory receptor cells. *Neuron*. 2011;71:389-405.
31. Agathocleous M, Harris WA. From progenitors to differentiated cells in the vertebrate retina. *Annu Rev Cell Dev Biol*. 2009;25:45-69.
32. Bassett EA, Wallace VA. Cell fate determination in the vertebrate retina. *Trends Neurosci*. 2012;35:565-573.
33. Young RW. Cell differentiation in the retina of the mouse. *Anat Rec*. 1985;212:199-205.
34. Nasonkin I, Mahairaki V, Xu L, et al. Long-term, stable differentiation of human embryonic stem cell-derived neural precursors grafted into the adult mammalian neostriatum. *Stem Cells*. 2009;27:2414-2426.
35. Lamba DA, Gust J, Reh TA. Transplantation of human embryonic stem cell-derived photoreceptors restores some visual function in Crx-deficient mice. *Cell Stem Cell*. 2009;4:73-79.
36. Hockemeyer D, Soldner F, Beard C, et al. Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. *Nat Biotechnol*. 2009;27:851-857.
37. Hockemeyer D, Wang H, Kiani S, et al. Genetic engineering of human pluripotent cells using TALE nucleases. *Nat Biotechnol*. 2011;29:731-734.
38. Mali P, Yang L, Esvelt KM, et al. RNA-guided human genome engineering via Cas9. *Science*. 2013;339:823-826.
39. Kaewkhaw R, Kaya K, Brooks M, Homma K, et al. Transcriptome dynamics of developing photoreceptors in 3-D retina cultures recapitulates temporal sequence of human cone and rod differentiation revealing cell surface markers and gene networks. *Stem Cells*. 2015;33:3504-3518.
40. Zou J, Maeder ML, Mali P, et al. Gene targeting of a disease-related gene in human induced pluripotent stem and embryonic stem cells. *Cell Stem Cell*. 2009;5:97-110.
41. Zou J, Mali P, Huang X, Dowey SN, Cheng L. Site-specific gene correction of a point mutation in human iPS cells derived from an adult patient with sickle cell disease. *Blood*. 2011;118:4599-4608.
42. Mellough CB, Collin J, Khazim M, et al. IGF-1 Signaling plays an important role in the formation of three-dimensional laminated neural retina and other ocular structures from human embryonic stem cells. *Stem Cells*. 2015;33:2416-2430.

43. Meyer JS, Howden SE, Wallace KA, et al. Optic vesicle-like structures derived from human pluripotent stem cells facilitate a customized approach to retinal disease treatment. *Stem Cells*. 2011;29:1206-1218.
44. Trapnell C, Cacchiarelli D, Grimsby J, et al. The dynamics and regulators of cell fate decisions are revealed by pseudotemporal ordering of single cells. *Nat Biotechnol*. 2014;32:381-386.
45. Akimoto M, Cheng H, Zhu D, et al. Targeting of GFP to newborn rods by Nrl promoter and temporal expression profiling of flow-sorted photoreceptors. *Proc Natl Acad Sci U S A*. 2006;103:3890-3895.
46. Postel K, Bellmann J, Splith V, Ader M. Analysis of cell surface markers specific for transplantable rod photoreceptors. *Mol Vis*. 2013;19:2058-2067.
47. Lakowski J, Gonzalez-Cordero A, West EL, et al. Transplantation of photoreceptor precursors isolated via a cell surface biomarker panel from embryonic stem cell-derived self-forming retina. *Stem Cells*. 2015;33:2469-2482.
48. Roger JE, Hiriyan A, Gotoh N, et al. OTX2 loss causes rod differentiation defect in CRX-associated congenital blindness. *J Clin Invest*. 2014;124:631-643.
49. Brooks MJ, Rajasimha HK, Roger JE, Swaroop A. Next-generation sequencing facilitates quantitative analysis of wild-type and Nrl(-/-) retinal transcriptomes. *Mol Vis*. 2011;17:3034-3054.
50. He L, Vasiliou K, Nebert DW. Analysis and update of the human solute carrier (SLC) gene superfamily. *Hum Genomics*. 2009;3:195-206.
51. Homma K, Okamoto S, Mandai M, et al. Developing rods transplanted into the degenerating retina of crx-knockout mice exhibit neural activity similar to native photoreceptors. *Stem Cells*. 2013;31:1149-1159.
52. Hambright D, Park KY, Brooks M, McKay R, Swaroop A, Nasonkin IO. Long-term survival and differentiation of retinal neurons derived from human embryonic stem cell lines in immunosuppressed mouse retina. *Mol Vis*. 2012;18:920-936.
53. Tsai Y, Lu B, Bakondi B, et al. Human iPSC-derived neural progenitors preserve vision in an AMD-like model. *Stem Cells*. 2015;33:2537-2549.
54. Yanai A, Laver CR, Gregory-Evans CY, Liu RR, Gregory-Evans K. Enhanced functional integration of human photoreceptor precursors into human and rodent retina in an ex vivo retinal explant model system. *Tissue Eng Part A*. 2015;21:1763-1771.
55. Zhou PY, Peng GH, Xu H, Yin ZQ. c-Kit+ cells isolated from human fetal retinas represent a new population of retinal progenitor cells. *J Cell Sci*. 2015;128:2169-2178.
56. Wu SM, Hochedlinger K. Harnessing the potential of induced pluripotent stem cells for regenerative medicine. *Nat Cell Biol*. 2011;13:497-505.
57. Phillips MJ, Perez ET, Martin JM, et al. Modeling human retinal development with patient-specific induced pluripotent stem cells reveals multiple roles for visual system homeobox 2. *Stem Cells*. 2014;32:1480-1492.
58. Singh R, Shen W, Kuai D, et al. iPSC cell modeling of Best disease: insights into the pathophysiology of an inherited macular degeneration. *Hum Mol Genet*. 2013;22:593-607.
59. Grieshammer U, Shepard KA. Proceedings: consideration of genetics in the design of induced pluripotent stem cell-based models of complex disease. *Stem Cells Transl Med*. 2014;3:1253-1258.
60. Desbordes SC, Placantonakis DG, Ciro A, et al. High-throughput screening assay for the identification of compounds regulating self-renewal and differentiation in human embryonic stem cells. *Cell Stem Cell*. 2008;2:602-612.
61. Lee G, Ramirez CN, Kim H, et al. Large-scale screening using familial dysautonomia induced pluripotent stem cells identifies compounds that rescue IKBKAP expression. *Nat Biotechnol*. 2012;30:1244-1248.
62. Welsbie DS, Yang Z, Ge Y, et al. Functional genomic screening identifies dual leucine zipper kinase as a key mediator of retinal ganglion cell death. *Proc Natl Acad Sci U S A*. 2013;110:4045-4050.
63. Watanabe K, Ueno M, Kamiya D, et al. A ROCK inhibitor permits survival of dissociated human embryonic stem cells. *Nat Biotechnol*. 2007;25:681-686.
64. Braam SR, Nauw R, Ward-van Oostwaard D, Mummery C, Passier R. Inhibition of ROCK improves survival of human embryonic stem cell-derived cardiomyocytes after dissociation. *Ann N Y Acad Sci*. 2010;1188:52-57.
65. Fritsche LG, Chen W, Schu M, et al. Seven new loci associated with age-related macular degeneration. *Nat Genet*. 2013;45:433-439, 439e1-439e2.
66. Fritsche LG, Fariss RN, Stambolian D, Abecasis GR, Curcio CA, Swaroop A. Age-related macular degeneration: genetics and biology coming together. *Annu Rev Genomics Hum Genet*. 2014;15:151-171.
67. Danno H, Michiue T, Hitachi K, Yukita A, Ishiura S, Asashima M. Molecular links among the causative genes for ocular malformation: Otx2 and Sox2 coregulate Rax expression. *Proc Natl Acad Sci U S A*. 2008;105:5408-5413.
68. Muranishi Y, Terada K, Inoue T, et al. An essential role for RAX homeoprotein and NOTCH-HES signaling in Otx2 expression in embryonic retinal photoreceptor cell fate determination. *J Neurosci*. 2011;31:16792-16807.