Review Article

Stem Cell INVESTIGATION

Strategies for retinal cell generation from human pluripotent stem cells

Lindsey S. Weed, Jason A. Mills

Center for Advanced Retinal and Ocular Therapeutics, F.M. Kirby Center for Molecular Ophthalmology, Scheie Eye Institute, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA 19104, USA

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Correspondence to: Jason A. Mills. Center for Advanced Retinal and Ocular Therapeutics, F.M. Kirby Center for Molecular Ophthalmology, Scheie Eye Institute, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA 19104, USA. Email: Millsja@mail.med.upenn.edu..

Abstract: Induced pluripotent stem cells (iPSCs) are specialized self-renewing cells that are generated by exogenously expressing pluripotency-associated transcription factors in somatic cells such as fibroblasts, peripheral blood mononuclear cells, or lymphoblastoid cell lines (LCLs). iPSCs are functionally similar to naturally pluripotent embryonic stem cells (ESCs) in their capacity to propagate indefinitely and potential to differentiate into all human cell types, and are devoid of the associated ethical complications of origin. iPSCs are useful for studying embryonic development, disease modeling, and drug screening. Additionally, iPSCs provide a personalized approach for pathological studies, particularly for diseases that lack appropriate animal models. Retinal cell differentiations using iPSCs have been successful in this regard. Several protocols to generate various retinal cells have been developed to maximize a specific cell type or, most recently, to mimic *in vivo* retinal structure and cellular environment. As differentiation protocols continue to improve we are likely to see an increase in our basic understanding of various retinal degenerative diseases and the utilization of iPSCs in clinical trials.

Keywords: Induced pluripotent stem cells (iPSCs); cell differentiation; retinal degeneration

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Introduction

Shinya Yamanaka's 2006 discovery that specialized adult cells could be reprogrammed into a stem cell-like state revolutionized our ability to study and develop disease-specific models (1). Induced pluripotent stem cells (iPSCs) are specialized self-renewing cells that are generated by exogenously expressing pluripotency-associated transcription factors in somatic cells such as fibroblasts, peripheral blood mononuclear cells, or lymphoblastoid cell lines (LCLs). Prior to this discovery, the only known naturally pluripotent cells were embryonic stem cells (ESCs). Ethical concerns regarding the origins of human ESCs (hESCs) made research and any subsequent applications of the cells challenging. iPSCs are functionally similar to hESCs in their capacity for indefinite self-renewal and potential to differentiate into all human cell types, eliminating the ethical burden and allowing for prior developmental assays to be applied across any genetic background interchangeably (2-6). Mouse and human pluripotent stem cells have been used for a variety of applications such as studying embryonic development, disease modeling, drug screening, and cell replacement strategies. iPSCs offer a personalized approach to pathological studies, and are particularly useful for diseases that lack appropriate animal models.

Mutations causing hereditary retinal degenerative diseases have been identified in >265 genes and affect all

Table 1 Retinal cell differentiation markers

Stage of development	Marker
Anterior neuroepithelium	PAX6, OTX2
Eye field	RAX, PAX6, LHX2, SIX3, SIX6
Optic vesicle	PAX6, MITF, VSX2
RPE progenitor	PAX6, MITF
Neuroretinal progenitor	PAX6, VSX2
Photoreceptor progenitor	CRX, RCVRN
Mature RPE	MITF, BEST1, ZO-1, CRALBP, PEDF, PMEL17
Mature cone photoreceptor	Cone arrestin, red-opsin, blue-green opsin
Mature rod photoreceptor	RHO, NRL
Retinal ganglion cell	BRN3, HuD
Müller glia	GLUL

RPE, retinal pigment epithelium; RHO, rhodopsin.

human retinal cell lineages (7). It is impossible to have an animal model for each affected gene, let alone all the possible mutations of a gene. iPSC technology provides a unique opportunity to generate personalized developmental and disease models by reprogramming a patient's skin or blood cells and differentiating them into the retinal cell of interest using one of many differentiation strategies first established using mouse or human ESCs. Translating these protocols for human iPSC (hiPSC) differentiation was essential for developing gene and autologous cell replacement therapies. This review will describe the strategies to generate retinal pigment epithelium (RPE), neural retinal progenitors and precursors, and mature neural retinal cells using monolayers or three-dimensional cultures of pluripotent stem cells.

In vivo eye development

Organogenesis of the vertebrate eye begins with a pair of evaginations of the neuroepithelium from the neural tube mediated by the retinal homeobox transcription factor, RAX (8-11). These diverticula extend from the diencephalon and form hollow bulbs known as the optic vesicles. At this stage, the progenitors of the neuroepithelium have the potential to differentiate into the inner neural retina or the outer RPE. These cells are characterized by the expression of RAX as well as other key regulators of eye field specification, PAX6, LHX2, SIX3, and SIX6 (12). Ubiquitous expression of the microphthalmia transcription factor (MITF) regulated by OTX2 in the optic vesicle controls the bipotentiality of the neuroepithelium (13,14). The subsequent downregulation of these two transcription factors by VSX2 in the distal region and extrinsic signaling of fibroblast growth factors (FGF) from the overlying ectoderm initiate domain specification and define the population of inner retinal progenitor cells (RPCs) (15-18). Presumptive RPE will continue to express MITF.

Interactions with the surface ectoderm and extraocular mesenchyme initiate a thickening of the ectoderm into the lens placodes, which cause an axial invagination of the distal portion of the optic vesicles to form the optic cups. These structures consist of an RPE and neural retinal progenitor bilayer of cells. RPCs then proliferate to form a stratified structure, eventually giving rise to lineage-restricted precursor cells that will mature into postmitotic retinal neurons and Müller glia. *Table 1* provides a list of retinal cell markers for each of these stages of development.

RPE differentiations

RPE is the most well-characterized retinal cell differentiation. The RPE is a monolayer of cells that structurally and metabolically supports the underlying neurosensory retina, including the photoreceptors, and forms part of the blood-retinal barrier. It functions mainly in nutrient and ion transport, phagocytosis of shed photoreceptor outer segments, light absorption, and vitamin A metabolism (19-23). Dysfunction of this specialized tissue can lead to photoreceptor dystrophy and blindness, such as that associated with age-related macular degeneration (AMD), the leading cause of irreversible blindness in the maturing population worldwide (24). RPE cells are most easily identified by their hexagonal geometry and pigmentation.

For more than a decade, RPE have been generated from pluripotent stem cells by spontaneous differentiate upon removal of basic fibroblast growth factor (bFGF) from the cell culture medium (25-32). Gene expression profiling asserted the quantitative similarity between RPE generated using this strategy and human fetal RPE. Initial pigmentation typically appeared 25-30 days after the start of differentiation, although this was cell-line dependent and can vary considerably between differentiations. Foci of pigmented cells large enough for dissection and enrichment occurred 60–90 days after bFGF depletion. These populations of cells have RPE hexagonal geometry,

Table 2 Transcription factors and small molecules commonly used in retinal cell differentiations

Differentiation Factor	Function in Retinal Differentiation
bFGF	Protein that maintains pluripotency in iPSC media and promotes neuroretinal differentiation
NIC	Amide of vitamin B3 that promotes neuralization
Activin A	TGF beta-like protein that maintains expression of pluripotency markers and shares downstream effector molecules with Nodal signaling that can promote neuralization
Noggin	Protein that induces anterior neural differentiation by inhibiting TGF-beta superfamily proteins, such as BMP
RA	Metabolite of vitamin A that promotes terminal differentiation of photoreceptors
Shh	Morphogen important for axis patterning and photoreceptor differentiation
IGF1	Protein that promotes eye field specification and retinal progenitor identity
Dkk1	Secreted agonist of the Wnt/β-catenin signaling pathway that induces anterior neuralization
LeftyA	Nodal signaling agonist that supports retinal progenitor specification
SB-431542	Nonbiological small molecule that selectively inhibits TGF-beta, activin, and nodal signaling pathways
iWR1e	Nonbiological small molecule that inhibits Wnt signaling to promote anterior neuralization
SAG	Nonbiological agonist of smoothened protein of hedgehog signaling pathway that supports neural proliferation
CHIR99021	Nonbiological small molecule that inhibits GSK3, a Wnt signaling pathway kinase
DAPT	Nonbiological agonist of notch signaling pathway to promote photoreceptor differentiation

FGF, basic fibroblast growth factor; iPSC, induced pluripotent stem cell; NIC, nicotinamide; BMP, bone morphogenic protein; RA, retinoic acid; Shh, sonic hedgehog; IGF1, insulin-like growth factor 1; Dkk1, dickkopf-related protein 1; SAG, smoothened agonist.

pigmentation, stain for classic protein markers such as Best1, ZO-1, MITF, and PEDF, have phagocytosis potential, and generate a transepithelial resistance (TER) comparable to fetal-derived RPEs. However, only after eight months in culture do cells start to express *RPE65*, a hallmark of terminally differentiated RPE.

The next attempt to increase differentiation kinetics of RPE involved the addition of nicotinamide (NIC) and Activin A to pluripotent cells (33-36). *Table 2* describes the function of these small molecules and others in retinal cell differentiations. Large numbers of pigmented clusters could be identified after only six weeks, significantly increasing RPE generation dynamics. Additional factors were introduced as retinal-inducing factors: noggin, retinoic acid, and sonic hedgehog (37-44). RPE generation was significantly increased when using MITF positive+ cells as a readout (37). More recently, emulating *in vivo* RPE development cues *in vitro* proved useful for rapid RPE-directed differentiation (45-47). Combined use of insulin-like growth factor 1 (IGF1), noggin, bFGF, and dickkopf-related protein 1 (Dkk1) with the serial application of NIC, Activin A, fibroblast growth factor receptor 1 (FGFR1) inhibitor SU5402, and vasoactive intestinal peptide (VIP) allowed for ~80% RPE generation efficiency with hESCs and ~60% efficiency with hiPSCs based on PMEL17 expression (45). Gene expression analysis revealed an accelerated ocular morphogenesis from early eye field to optic vesicle and RPE when compared to *in vivo* development. Sheets of RPE expressing critical RPE markers could be obtained in as little as 14 days and homogenous cultures of RPE produced in three weeks.

Maruotti *et al.* described an alternative approach to hiPSC-RPE differentiation utilizing small molecules which are favored for potential clinical applications over growth factors derived from animal or bacterial cells, which are liable to vary from lot-to-lot and escalate the potential for patient infection or immune rejection. Small-molecule chetomin (CTM) was identified in a high-throughput qPCR screen to consistently upregulate RPE markers MITF, OTX2, and PMEL17 (48). CTM is a metabolite of the fungus *Chaetomium* species that inhibits the transcriptional activation of the hypoxia-inducible factor (HIF) pathway (49). In combination with NIC, CTM potently induces directed-RPE differentiation in multiple hESC and hiPSC lines as measured by marker expression. Typical RPE morphology is achieved by switching hESC medium to RPEM two weeks post cotreatment with NIC

and CTM. Pure monolayers of functional RPE can be obtained following a single passage of these cultures.

Although many recent publications highlight the advances in RPE differentiation protocols, substantial sources of variability in the efficiency of RPE generation include cell line, genetic background, passaging method, passage number, seeding density, and extracellular matrix (50). All of these models have been valuable for the generation of *in vitro*-derived RPE that have been used for phase I clinical trials for the treatment of macular degeneration (51). Reproducible differentiations are paramount for the clinical success of hiPSC-RPE based therapies.

Adherent neural retinal differentiations

The RPCs of the neuroblastic layer of the optic cup divide symmetrically during early development to increase the progenitor pool size in preparation to give rise to the seven different mature cell types of the neural retina – retinal ganglion cells, amacrine cells, bipolar cells, horizontal cells, rod photoreceptors, cone photoreceptors, and Müller glia (52). This process is coordinated in time and space by both intrinsic and extrinsic factors (53). Eventually the progenitor pool begins to divide asymmetrically to generate daughter cells that can adopt differing fates during development (54). Differentiation into specific post-mitotic precursor cells is initiated upon termination of proliferation. These precursors then migrate towards the prospective layer of the neural retina in which the mature cell will reside (55). Multipotent RPCs and the precursors that they give rise to are of particular interest for therapeutics due to the number of degenerative conditions the neural retina is subject to.

Inherited retinopathies, such as retinitis pigmentosa (RP), Leber congenital amaurosis (LCA), and Stargardt disease (STGD), are a genetically and phenotypically heterogeneous group of monogenic diseases that mainly effect genes expressed in the light-sensitive rod and cone photoreceptors. Mutations in more than 250 identified genes can affect almost every aspect of photoreceptor function causing diverse pathophysiologies that culminate in cell death (56). Alternative pathogenic variants of the same gene can cause multiple disparate retinal diseases (7). This complex etiology necessitates the need for iPSC technology for multiple reasons. Often an affected gene or specific mutation will lack a corresponding animal model, making pre-clinical treatment studies difficult or impossible to extrapolate to humans. hiPSCs provide a unique opportunity to generate personalized models of disease to be used in a variety of studies, from determining pathology to demonstrating intervention efficacy (57). Additionally, they are a source of autologous cells for transplantation. The generation of RPCs and further retinal lineages has been performed using either adherent cell aggregations into spheroid structures or densely packed monolayer cultures.

In vitro differentiation to RPCs and photoreceptor precursors requires several sequential steps beginning with the induction of an anterior neural fate by inhibition of Wingless (Wnt) and bone morphogenic protein (BMP) (58-62). Three week culturing of adherent cell aggregates, known as embryoid bodies (EBs), of the H-1 hESC line with potent Wnt and BMP inhibitors Dkk1 and noggin, respectively, IGF-1, and bFGF exhibit increased expression of eye field transcription factors (EFTFs) and retinal progenitor markers in comparison to undifferentiated and factor-free cultures (63). Immunofluorescence labeling with specific retinal neuron antibodies revealed a preference for ganglion and amacrine precursor cells. While markers for immature photoreceptors, CRX and NRL, were also expressed in approximately 12% of cells, very few mature photoreceptor markers were present in the cultures. Similar results were obtained with hiPSC using this method (64).

Based on differentiations with mouse and monkey ESCs, initial treatment of EBs of the khES-1 hESC line with Dkk1 and Nodal antagonist, LeftyA, resulted in approximately 16% $RX^+/Pax6^+$ colonies after 35 days in culture (65). Nodal is a member of the transforming growth factor β superfamily. Upon addition of retinoic acid and taurine, factors that can promote the terminal differentiation of photoreceptors, CRX^+ cells began to appear in culture at 90 days and increased as the culture period was extended to 120 (~11% of total cells) and 170 (~20% of total cells) days. Rhodopsin $(RHO)^+$ rod photoreceptors were present by day 130 and also increased in number to ~8.5% of total cells by day 200. Red/green opsin $(OPN1LW)^+$ or blue opsin $(OPN1SW)^+$ cells could also be found in close proximity to RHO^+ cells. The results were replicated with the khES-3 hESC line. These putative photoreceptors expressed additional genes involved in phototransduction. Although time-consuming and inefficient, this study used a chemically defined culture medium which has better potential for clinical application. Later differentiations utilizing nonbiological small-molecules Y-27632, CKI-7, and SB-431542 to inhibit Wnt and Nodal signaling made greater strides towards xeno-free culture systems, but yielded a lower percentage of RHO+ cells than cultures treated with

Dkk1 and LeftyA (66).

Mellough *et al.* combined some aspects of these two previous studies with additional modifications to mimic neural and retinal development in an attempt to design a photoreceptor differentiation protocol for hESC and hiPSC with higher yields and less culture time (67). The three-step differentiation resulted in 16% of CRX^+ cells and the appearance of late-stage photoreceptor-specific markers RHO, OPN1LW, and OPN1SW by day 45, representing a dramatic decrease in time to differentiation from previous reports (65). However, the population of photoreceptor marker positive cells rapidly declined by day 60. Interestingly, control populations differentiated under basal media conditions supplemented only with B27 supplement and, at later time points, B27 and N2 supplements, exhibited neuronal, eye field, retinal, and photoreceptor gene expression, revealing the importance of B27 and N2 in serum-free media conditions for photoreceptor generation within the shortened culturing time frame. Both B27 and N2 are chemically defined blood serum substitutes consisting of multiple hormones, including insulin and progesterone, and other growth factors that promote neuronal cell survival (68-71).

To date, the protocol that yields that highest amount of photoreceptors from hESCs in the shortest amount of time utilizes a human recombinant protein known as COCO, a multifunctional product of the Cerberus gene family expressed in the developing and adult mouse retina that inhibits BMP, TGFβ, and Wnt signaling pathways (72). COCO has also been shown to antagonize other ligands of the TGF-beta superfamily (which includes BMP and TGFβ) with roles in retinal development, Nodal and Activin (73,74). In combination with bFGF and IGF1 treatment, the H9 hESC line differentiated to induce expression of early retinal and photoreceptor genes *SIX6* and *CRX* and phototransduction gene *OPN1SW* ~10-fold more effectively than with noggin and Dkk1 instead of COCO after 21 days of culture (75). S-opsin was expressed by ~70% of differentiated cells at this time point. The additional blocking of TGFβ signaling appears to be paramount to the success of this protocol as compared to previous methods that inhibit only BMP and Wnt or Wnt and Nodal signaling (63-67). These findings also suggest that antagonizing all of these signaling pathways is required for cone genesis at the expense of rod genesis, which measured by marker expression appeared to be the more prevalent photoreceptor in the retinal cultures of the previously established protocols.

Three-dimensional organoids

Perhaps the most relevant model for studying inherited retinal disease pathologies and their potential treatments is the 3D-stratified retinal organoids first developed with mouse ESCs and later recapitulated with hESCs (76-79). hESCs dissociated into single cells are reaggregated in low-cell-adhesion V-bottom 96-well plates at a density of 9,000–12,500 cells/well in retinal defined differentiation medium containing ROCK inhibitor (Y-27632) and Wnt inhibitor (iWR1e). Matrigel is added to the culture medium shortly following initial plating as a basement membrane for structural development of epithelial character. Addition of fetal bovine serum (FBS) and hedgehog signaling pathway smoothened agonist (SAG) culminate in more than 70% of total cells expressing early retinal marker, RAX (77). These conditions favor the formation of $VSX2^+$/$PAX6^+$ neural retina fated cells, but treatment with Wnt agonist CHIR99021 during days 18-21, after the cells have committed to a retinal fate, could efficiently induce swaths of $MITF^+$ presumptive RPE without disturbing VSX2 expression in the distal portion of the retinal epithelium to be more representative of developing optic cups *in vivo*.

The autonomous appearance of evaginations reminiscent of optic vesicles and their subsequent invagination to form optic cups from homogenous hESC aggregates observed in these protocols appears to closely mimic the complex tissue interactions of *in vivo* retinogenesis (80). However, these structures develop at low efficiency and their successful dissection is what determines the retinal organoid yield, which can be quite limited. Emitting dissection risks compromising the retinal identity of organoids with integration of non-retinal structures (81,82). Völkner *et al.* provide a protocol for unbiased neuroepithelium trisection at the eyefield stage to produce high numbers of large, stratified organoids committed to retinal fate (79). Another modification to the Nakano *et al.* protocol that may increase the differentiation efficiency to retinal progenitors and neural retina within individual organoids is the lowering of oxygen tension from atmospheric 20% to 2%, which is more representative of the physiologic oxygen level during human organogenesis. After 21 days in a 2-dimensional culture system, a greater number of hiPSC-RPCs and hESC-RPCs were $PAX6^+$/$VSX2^+$ in 2% oxygen compared to 20% oxygen (83). qPCR analysis also revealed elevated expression of PAX6 and VSX2 in RPCs kept at 2% oxygen. In contrast, optic vesicles are maintained in 40% oxygen long-term (77,79). *In vitro* differentiation efficiency may

benefit from replicating *in vivo* conditions, but the ability to preserve multilayered organoids without a vascular supply of nutrients requires higher oxygen tension and organoid agitation (84).

Maturation of hESC-derived optic cups over several weeks (day 47–120) leads to the differentiation of CRX+/RECOVERIN+ photoreceptors (79). This process can be accelerated with inhibition of Notch signaling by DAPT (76,77,79,85). However, DAPT treatment appears to favor cone photoreceptor genesis over rod photoreceptor genesis, consistent with reports of Notch suppression of cone fate specification (86,87). The presence of retinal ganglion and amacrine cells can be detected by day 37, while horizontal, ON bipolar, and Müller glial cells appear in small numbers after 90 days in culture (78). Retinal organoidogenesis with hiPSCs has been extremely limited. Parfitt *et al.* were successful at generating hiPSC-derived RECOVERIN+ optic cups by 13 weeks in culture based on the Nakano *et al.* differentiation and dissection protocol (77,88). Expression of mature photoreceptor markers appeared after 21 weeks in culture. A BRN3+/HuD+ ganglion layer was the only other mature neural retinal cell type observed, suggesting that the variance in timing and reproducibility with which retinal organoids generate all major retinal cell types remains a challenge.

Conclusions

The ability to differentiate iPSC into the various cell types of the retina has been useful for studying early retinal development and etiology, screening small compounds, testing gene replacement therapies, and providing source material for cell replacement therapies. Several protocols to generate various retinal cells have been developed to maximize a specific cell type or follow good manufacturing practice (GMP) guidelines for xeno-free therapeutic culture systems. Different retinal cells may be generated using varying protocols suitable for the intent of a particular study. While the efficiency at which these cells can be generated has increased significantly since the first spontaneous differentiations, several challenges still exist. For one, isolating pure cell populations from neural retinal differentiations remains difficult. Secondly, not all cell types or cell structures are generated with equal efficiency. Muller glia, the last cell type to differentiate during *in vivo* retinogenesis, appear in low yields after ~90–180 days in 3D-organiod culture. Additionally, only ~3% of cells that stain positive for mature photoreceptor markers develop outer segments after ~160 days in culture. As differentiation protocols continue to improve we are likely to see an increase in our basic understanding of various retinal degenerative diseases and the utilization of iPSC in clinical trials.

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Footnote

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