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SPECIAL ISSUE

Strategies for Restoring Sight in Retinal Dystrophies

REVIEW ARTICLE

Lab generated retina: Realizing the dream

CARLA B. MELLOUGH,[1] JOSEPH COLLIN,[1] EVELYNE SERNAGOR,[2] NICHOLAS K. WRIDE,[1,3] DAVID H.W. STEEL,[1,3] AND MAJLINDA LAKO[1]

[1]Institute of Genetic Medicine, Newcastle University, Newcastle, United Kingdom
[2]Institute of Neuroscience, Newcastle University, Newcastle, United Kingdom
[3]Sunderland Eye Infirmary, Sunderland, United Kingdom

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Abstract

Blindness represents an increasing global problem with significant social and economic impact upon affected patients and society as a whole. In Europe, approximately one in 30 individuals experience sight loss and 75% of those are unemployed, a social burden which is very likely to increase as the population of Europe ages. Diseases affecting the retina account for approximately 26% of blindness globally and 70% of blindness in the United Kingdom. To date, there are no treatments to restore lost retinal cells and improve visual function, highlighting an urgent need for new therapeutic approaches. A pioneering breakthrough has demonstrated the ability to generate synthetic retina from pluripotent stem cells under laboratory conditions, a finding with immense relevance for basic research, *in vitro* disease modeling, drug discovery, and cell replacement therapies. This review summarizes the current achievements in pluripotent stem cell differentiation toward retinal cells and highlights the steps that need to be completed in order to generate human synthetic retinae with high efficiency and reproducibly from patient-specific pluripotent stem cells.

Keywords: Neural retina, Retinal pigmented epithelium, Human pluripotent stem cells, Differentiation, Age related macular degeneration (AMD), Hereditary retinal dystrophies (HRDs)

Background

Blindness has a significant impact on the quality of a person's life, often resulting in depression, social isolation, and premature death. This poses a major burden to society due to lost productivity and earnings as well as the cost of treatment, rehabilitation, education of the visually impaired and provision of visual aids. Recent estimates indicate that the global number of people with sight loss is 285 million, of whom 39 million are classified blind (Pascolini & Mariotti, 2012). Visual impairment is not evenly distributed across age groups, with more than 82% of blindness occurring beyond 50 years of age. Although the prevalence of blindness in children is approximately 10 times lower than in adults, childhood blindness remains a high priority because the predicted duration of their suffering is protracted (Pascolini & Mariotti, 2012). A conservative analysis estimated that the loss of productivity of individuals with visual impairment in 2003 bore a $42 billion impact on society with a projected rise to $110 billion by 2020; hence, the return on investment is potentially high compared with the research costs involved in the development of new treatment modalities.

Address correspondence to: Majlinda Lako, Ph.D., Newcastle University, Institute of Genetic Medicine, International Centre for Life, Newcastle NE1 3BZ, United Kingdom. E-mail: majlinda.lako@ncl.ac.uk

Diseases affecting the outer retina including age related macular degeneration (AMD) and hereditary retinal dystrophies (HRDs) account for approximately 26% of blindness worldwide. The incidence is expected to double by 2020 and to increase to 75% by 2040 as the world population steadily ages (Pascolini & Mariotti, 2012). Although in some cases the primary pathological event originates in the retinal pigmented epithelium (RPE, a supportive monolayer of pigmented cells forming part of the blood-retinal barrier), the final impact of both AMD and HRDs is the loss of photoreceptors. While there are a number of agents (including high dose antioxidants, neuronal survival agents, and vascular endothelial growth factor [VEGF] inhibitors in patients with neovascular AMD: Krishnadev et al., 2010; Arias, 2010; Kaiser et al., 2007; Rosenfeld et al., 2006) that have been shown to slow outer retinal disease progression, there are no treatments to restore photoreceptors that have already been lost; hence, there is a pressing need for research into the replacement and/or reactivation of dysfunctional photoreceptors as well as RPE in order to restore visual function in these cases. The eye is well suited for the development of cell transplantation therapies as it is easily accessible, allowing the accurate delivery of cells to the retina with minimal risk of systemic effects. To date, gene therapy has dominated the majority of clinical trials for the treatment of eye disease (Otani et al., 2004; Bainbridge et al., 2008; Maguire et al., 2008; Smith et al., 2009; Cideciyan et al., 2013); however, this approach is applicable only to HRDs that are well

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characterized genetically, show early onset symptoms and slow degeneration. Early treatment of such forms of HRD by gene therapy is likely to succeed in both improved visual function and photoreceptor protection; however, at later stages of the disease this method is unlikely to be effective and will necessitate additional approaches combined with gene therapy (Cideciyan et al., 2013). In such cases, cell replacement therapies offer an attractive complementary approach.

Pluripotent is best: Why and how?

A key reason for using stem cell–based therapies to treat retinal disorders is the prospect of generating unlimited quantities of desired cell types for transplantation. A variety of cell types have been tested in animal models and human clinical trials (Table 1) for their ability to repopulate the degenerate retina or rescue retinal neurons from further degeneration, in particular the RPE. In most cases, these sources include autologous cells from the eye itself (iris pigmented epithelial cells [IPE] and RPE). As can be seen from Table 1, both autologous RPE transplantation and retinal relocation surgery to a healthy area of RPE can result in increase in visual acuity or stabilization of vision in a proportion of patients; however, postoperative complications and the likelihood of continued deterioration of visual acuity secondary to disease recurrence are also quite high, making these procedures unlikely to succeed in a large number of patients with AMD or HRD. Nevertheless, they provide proof of concept that diseased RPE replacement, probably most optimally performed in a sheet configuration, can result in visual improvement and photoreceptor rescue in some cases.

Neurosensory retinal regeneration is more complex. Transplants of hematopoietic and mesenchymal stem cells isolated from adult bone marrow are being tested in patients with retinitis pigmentosa (RP), Stargardt's disease and AMD, and the clinical outcomes are eagerly awaited (Table 1). Notwithstanding this, studies conducted in animal models have demonstrated that these cells are unable to repopulate the degenerate retina and give rise to functional photoreceptors, but act to prevent further retinal degeneration *via* the production of neurotrophic factors. Therefore, although this approach may be useful for retinal protection during the early degenerative phase, it is unlikely to work in cases of advanced degeneration where substantial retinal remodeling has already taken place. Other studies have investigated the possibility of deriving photoreceptor cells by reprogramming cell sources found within the eye, either using genetic manipulation or coaxing cell plasticity by manipulating culture conditions. To this end, various ocular tissues have been tested including RPE (Li et al., 2010), IPE (Haruta et al., 2001), ciliary body (MacNeil et al., 2007) and limbal epithelium (Zhao et al., 2008). Of these, RPE cells appear the most promising to date with experimental findings showing that reprogramming of chick RPE with neurogenin can give rise to cell layers expressing photoreceptor and phototransduction genes (Li et al., 2010). Functional assays such as the response to light and 9-cis-retinal were however not performed, nor transplantation tests, thus leaving open the question whether full reprogramming of RPE to functional photoreceptors had taken place.

More recently, pluripotent stem cells have been investigated for their ability to contribute to retinal restoration. The term pluripotent stem cell is commonly used to describe two types of stem cells characterized by their indefinite self-renewal ability and the capacity to give rise to any somatic cell type in the adult, namely embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) (Mellough et al., 2009). Human ESCs (hESCs) are derived from surplus *in vitro* fertilized embryos and have been widely used in the last decade as a research tool to understand the mechanisms behind the maintenance of pluripotency, human embryonic development and congenital disease. The use of human embryos for research purposes is surrounded by a number of ethical issues, prohibiting hESC derivation and application in several countries. In addition, a major issue related to their biological application is the evidence that their differentiated progeny expresses human leukocyte antigens (HLAs) that could result in graft rejection. This could be overcome by the creation of HLA-typed hESC banks, from which a best match can be selected for each transplant recipient (Taylor et al., 2005). However, human iPSCs (hiPSCs) bypass both of these issues as they are generated by reprogramming somatic cells back to a pluripotent-like state akin to ESCs (Lako et al., 2010; Rashid & Vallier, 2010). As such, these cells share many key characteristics of hESCs including the ability to proliferate indefinitely and differentiate into different cell types, but also represent a source of autologous stem cells given that they can be derived from the patients themselves. Such patient-derived cells present a unique opportunity to create *in vitro* disease models, which can be exploited to understand disease pathology and drug discovery (see Fig. 1). This becomes extremely important for degenerative diseases such as those affecting the retina, where availability of patient-specific cells (i.e., photoreceptors and RPE) is only possible with invasive surgery or *post mortem*. New tools developed in the gene therapy field including improved and safer viral vectors as well as the possibility of correcting endogenous mutations through the application of site-specific restriction endonucleases, such as Zinc Finger Nucleases (ZFNs) and Transcription Activator-Like Effector Nucleases (TALENs), also mean that functional cells for transplantation can be produced from hiPSCs that, without genetic modification techniques, would otherwise harbor typical disease characteristics and therefore be less suitable for transplantation (Sundaram et al., 2012; Collin & Lako, 2011).

The choice of cell type for transplantation into the neurosensory retina is heavily dictated by the ability of grafted cells to integrate with high efficiency within the correct retinal layer and contribute to improved vision by restoring retinal circuitry. Studies performed by Robin Ali's group in murine retina have shown that postmitotic photoreceptor precursors isolated at a very specific time point during mouse embryonic development (MacLaren et al., 2006; Lakowski et al., 2010) demonstrate the highest levels of engraftment within host retinae and acquire the specialized morphological features of mature photoreceptors. For the transplants to be successful, other events have to occur in addition to photoreceptor cell engraftment into the correct retinal layer. An important step is the ability of newly integrated cells to make the appropriate synaptic connections with the host retina and to restore functional retinal circuitry to a measurable degree that can be demonstrated by different functional tests. A recent study (Gonzalez-Cordero et al., 2013) has shown that newly transplanted rod photoreceptor precursors isolated from postnatal mice form typical triad synaptic connections with second-order bipolar and horizontal cells of the recipient retina and generate visual signals which are transmitted to higher visual areas in the brain. Improved vision was shown in transplanted mice, as assessed by optomotor head-tracking responses and a visually guided water-maze test, thus demonstrating the feasibility of photoreceptor transplantation as a therapeutic strategy. Notwithstanding these remarkable results, one has to be aware of the multiple challenges on the route to restoring functional retinal circuitry by transplanted photoreceptors, not the least of which is the ability to achieve a sufficient critical mass of engrafted photoreceptors (Gonzalez-Cordero et al., 2013). In addition, changes in the host retinal environment such as glial scarring and the integrity of the outer

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Table 1. *A summary of clinical trials focusing on stem cell transplantation in AMD and HRD patients*

Patient group	Number (eyes)	Surgery	Cell source	Outcomes/institution or company with start date	References
Neovascular AMD	>100	Choroidal neovascular membrane (CNVM) removal with subretinal sheet transplant	Autologous RPE sheet	Short-term visual acuity gain in 25–60% but long-term decline in vision reported and high complication rate requiring repeat surgery in 10–35%	(van Zeeburg et al., 2012; Chen et al.,2009; MacLaren et al., 2007; MacLaren et al., 2005; Binder et al., 2004; Falkner-Radler et al., 2011)
Neovascular AMD	>100	Retinal relocation surgery with retinal rotation after 360° retinotomy	Relocation of neurosensory retina to non-macular area of RPE	Visual improvement in 25–60% but long-term decline in vision reported and high complication rate requiring repeat surgery in 10–35%	(Eckardt et al., 1999; Aisenbrey et al., 2002; Pertile & Claes, 2002; Abdel-Meguid et al., 2003; Mruthyunjaya et al., 2004; Wong et al., 2004; Yamada et al., 2010)
Neovascular and dry AMD	9	Excision of CNVM with sheet transplant	Fetal RPE sheet	No significant visual improvement and rejection	(Algvere et al., 1999)
Neovascular AMD	12	Excision of CNVM and insertion of RPE sheet	Allogenic adult RPE cells in gelatin sheet	No visual improvement	(Tezel et al., 2007)
Neovascular AMD	~100	Surgical removal of CNVM combined with injection of cells subretinally	Suspension of autologous IPE cells	Visual stabilization achieved in most patients but no convincing improvement demonstrated	(Aisenbrey et al., 2006)
Dry AMD and RP	10	Subretinal insertion of sheet	Sheet of combined fetal RPE and retina	Short and longer term visual improvement reported	(Radtke et al., 2008)
Late and early stage RP	65 and 68	Intravitreal insertion of capsule	Human RPE cells contained in a small capsule secreting ciliary neurotrophic factor	Neurotech Pharmaceuticals, United States. Neither study reached primary endpoint of visual improvement	NCT00447993 phase II/III (Birch et al., 2013)
RP	5 planned	Intravitreal injection	Autologous bone marrow stem cells	University of San Paulo, Brazil: started in May 2009	NCT01068561 phase I
RP	10 planned	Intravitreal injection	Autologous bone marrow-derived mesenchymal stem cells	Mahidol University, Thailand: started in February 2012	NCT01531348 phase I
RP	15 planned	Not stated	Bone marrow and umbilical cord-derived mesenchymal stem cells	Chaitanya Hospital, India: to start soon	NCT01914913 phase I/II
RP, dry AMD, diabetic maculopathy, and retinal vein occlusions	15 planned	Intravitreal injection	Autologous CD34+ bone marrow stem cells	University of California, United States: started in April 2013	NCT01736059 phase I
Neovascular and dry AMD	10 planned	Intravitreal injection	Autologous CD34+ bone marrow stem cells	University of San Paulo, Brazil: started in January 2012	NCT01518127 phase I/II
Advanced dry AMD and Stargardt's macular dystrophy	12 planned	Subretinal injection	hESC-derived RPE cells in suspension	CHA Bio and Diostech, Republic of Korea: started in September 2012	NCT01674829 and NCT1625559 phase I/II
Stargardt's macular dystrophy and dry AMD	16 planned in each group	Subretinal injection	hESC-derived RPE in suspension	Advanced Cell Technology, United States and United Kingdom: started in January 2011	NCT01469832 and NCT01344993 phase I/II
Acute wet AMD and rapid vision decline	10 planned	Excision of CNVM and insertion of RPE sheet	hESC-derived RPE cells immobilized as a 6 mm by 3 mm sheet on a polyester membrane	Pfizer, United Kingdom: starting in August 2013	NCT01691261 phase I
Wet AMD	6 planned	Excision of CNVM and insertion of RPE sheet	Autologous hiPSC-derived RPE as a 1.3 mm by 3 mm sheet without a carrier	Kobe, Japan: started in September 2013	Phase I (Riken, 2013)

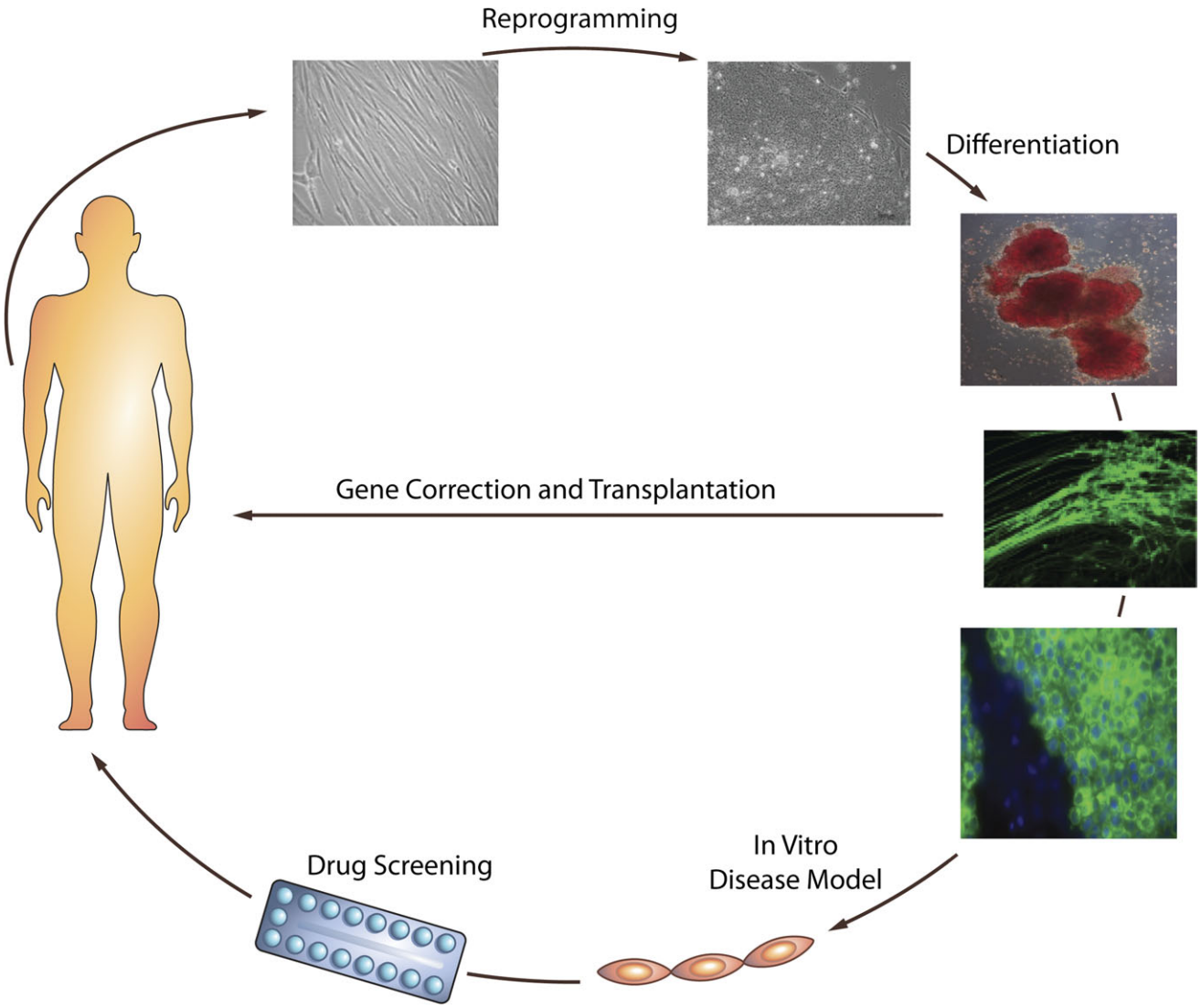


Fig. 1. Schematic summary of hiPSC derivation and its applications in disease modeling, drug discovery, and cell replacement therapies.

limiting membrane (which acts as a barrier to integration) differ with the type and stage of disease and, as shown in a recent paper, bear a heavy impact on photoreceptor transplantation (Barber et al., 2013), thus suggesting that both need to be fully assessed and manipulated to achieve the best photoreceptor transplantation across a wide range of retinal diseases at both early and late stages of degeneration.

All the above studies have been achieved in mouse and for this success to be translated to humans, equivalent cells would need to be isolated from second trimester fetuses, an issue which raises obvious ethical concerns. Multiple reports have shown that *in vitro* differentiation of hESCs and hiPSCs can in fact recapitulate many aspects of human embryonic development (Lako et al., 2010; Rashid & Vallier, 2010). Therefore, if human embryonic retinal precursors represent the optimal transplantable tissue for retinal restoration, then *in vitro* differentiation of hESCs and hiPSCs provides the best currently available platform to achieve this goal.

The earliest indication of the possibility of replicating the murine data using human-derived cells came from the first report of the production of human RPE during the spontaneous differentiation of hESCs in 2004 (Table 2; Aoki et al., 2009; Barber et al., 2013) and the presence of optic cup-like structures containing primitive neural retina and RPE in teratomas formed from pluripotent stem cells. With an aim to enhance the differentiation process and produce functional photoreceptors and RPE from hESCs and hiPSCs, several researchers in the field have used a number of growth factors and morphogens that are known to enhance anterior neural specification (bone morphogenetic protein [BMP] and Wnt inhibitors: Osakada et al., 2008; Hirami et al., 2009; Mellough et al., 2012; Lund et al., 2006), retinal precursor cell (RPC) emergence (insulin-like growth factor 1 [IGF-1]: Lund et al., 2006; Lamba et al., 2006; Mellough et al., 2012), photoreceptor maturation (activin A, taurine, sonic hedgehog [Shh]: Mellough et al., 2012), or RPE differentiation (nicotinamide, activin A: Idelson et al., 2009; refer to Table 2). Each of these studies was performed by differentiating cells as a monolayer under two-dimensional (2D) conditions and, despite being able to generate photoreceptor-like cells with high efficiency (up to 80% (Mellough et al., 2012), upon transplantation into animal models of retinal degeneration, such hESC-derived photoreceptors engrafted with low efficiency (an average of 3000 cells Nrl^+ human cells found in the Crx–/– retina after transplantation) failed to develop photoreceptor outer segments (an essential component for the phototransduction process) and only in very rare cases acquired the expression of photoreceptor markers (Lamba et al.,

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Table 2. *Summary of main protocols used to date to cue differentiation of hESC and hiPSC to RPE and photoreceptor cells*

Cell type	Differentiation method	Cell type obtained	Length of differentiation process	Engraftment in animal models	References
hESC	Monolayer/spontaneous differentiation	RPE	4–8 weeks	NA	(Barber et al., 2013)
hiPSC	Monolayer/spontaneous differentiation	RPE	6–8 weeks	NA	(Klimanskaya et al., 2004)
hESC	Floating embryoid bodies plated in coated tissue culture surfaces in media containing IGF-1, DKK-1, and noggin	RPCs	1–3 weeks	hESC-derived RPCs settle into wild type and Crx−/− adult mouse retina, differentiate into photoreceptors but fail to extend inner and outer segments	(Lund et al., 2006; Lamba et al., 2009)
hESC	Monolayer/spontaneous differentiation	RPE	2–7 weeks	Transplantation into RCS rats indicated survival of hESC-derived RPE cells	(Vugler et al., 2008)
hESC	Co-culture with PA6 stromal cells	RPE and RPCs	3–4 weeks	NA	(Gong et al., 2008)
hESC	Floating embryoid bodies followed by plating onto coated tissue culture surfaces. First induction media contained DKK-1, LEFTY-A; various other supplements added during the differentiation process	RPE and RPCs	120–200 days	NA	(Osakada et al., 2008)
hiPSC	Floating embryoid bodies followed by plating onto coated tissue culture surfaces. First induction media contained DKK-1, LEFTY-A; various other supplements added during the differentiation process	RPE and RPCs	120–200 days	NA	(Hirami et al., 2009)
hESC	Floating embryoid bodies followed by plating onto coated tissue culture surfaces. First induction media contained nicotinamide			Transplantation into RCS rats indicated survival of hESC-derived RPE cells	(Idelson et al., 2009)
hESC	Direct transplantation of hESC into adult host mouse eyes	Optic cup-like structures	5 weeks	NA	(Aoki et al., 2009)
hESC and hiPSC	Monolayer/spontaneous differentiation	RPE	2–7 weeks	Transplantation into RCS rats indicated survival of hESC/hiPSC-derived RPE, preservation of ONL, and improvement in visual acuity	(Carr et al., 2009)
hESC and hiPSC	Monolayer/spontaneous differentiation	RPE	4–12 weeks	NA	(Buchholz et al., 2009)
hESC and hiPSC	Floating embryoid bodies followed by plating onto coated tissue culture surfaces in chemically defined media. This was followed by a further step of rosette dissection and suspension culture	RPE and RPCs	10–17 weeks	NA	(Meyer et al., 2009)
hESC	3D culture of hESC-derived RPE and neural progenitor cells	RPCs and RPE	12 weeks	NA	(Nistor et al., 2010)
hESC and hiPSC	Monolayer culture in media containing DKK-1, IGF-1, and noggin	RPE and RPCs	3–8 weeks	Subretinal transplantation into adult mice indicated survival and engraftment within the outer nuclear layer (ONL), albeit at low efficiency	(Lamba et al., 2010)

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Table 2. Continued.

Cell type	Differentiation method	Cell type obtained	Length of differentiation process	Engraftment in animal models	References
hESC and hiPSC	Floating embryoid bodies followed by plating onto coated tissue culture surfaces in chemically defined media. This was followed by a further step of rosette dissection and suspension culture	Optic like vesicles containing RPCs	10–17 weeks	NA	(Meyer et al., 2011)
hiPSC	Floating embryoid bodies followed by plating in coated tissue culture surfaces in chemically defined media. This was followed by a further step of rosette dissection and suspension culture	Laminated neural retina containing photoreceptor like cells which are able to synapse	10–17 weeks	NA	(Phillips et al., 2012)
hESC and hiPSC	Monolayer/defined media	RPE	2 weeks		(Buchholz et al., 2013)
hiPSC	Monolayer/spontaneous differentiation	RPE	4 weeks	Transplantation into $Rpe65^{rd12}$/$Rpe65^{rd12}$ indicated survival of hiPSC-derived RPE and improved the ERG response	(Li et al., 2012)
hESC and hiPSC	Floating embryoid bodies followed by plating in coated tissue culture surfaces in chemically defined media supplemented with various growth factors and morphogens at different stages of differentiation	RPCs and RPE	6–9 weeks	NA	(Mellough et al., 2012)
hESC and hiPSC	Floating embryoid bodies followed by plating in coated tissue culture surfaces in xeno-free media	RPE	2–3 weeks	NA	(Vaajasaari et al., 2011)
hESC and hiPSC	Monolayer/spontaneous differentiation in the presence of different ECM components	RPE	5–6 weeks	NA	(Rowland et al., 2013)
hESC	3D culture of floating embryoid bodies in media containing FBS, KSR, Shh antagonist, Wnt and Rho kinase inhibitor and matrigel	Self-formed optic cups which undeform further differentiation into multilayered neural retina	4–5 weeks		(Nakano et al., 2012)
hESC and hiPSC	3D floating embryoid bodies in bioreactors in minimal media containing different supplements during differentiation process (N2, B27, matrigel, heparin, and Rho kinase inhibitor)	Cerebral organoids containing laminated neural retina	3–8 weeks	NA	(Lancaster et al., 2013)

2009), suggesting that 2D culture conditions may not be the ideal route for generating fully mature functional photoreceptors. What is fascinating is that although unable to elaborate outer segments following subretinal transplantation into Crx–/– mice, in the same study, hESC-derived retinal cells grafted into normal adult mouse retina were capable of outer segment formation, indicating that existing host photoreceptors or interphotoreceptor matrix may provide vital support for the final functional maturation of this cell type upon transplantation (Lamba et al., 2006).

Recent ground-breaking work from Yoshiki Sasai's group has shown that both murine ESCs (mESCs) and hESCs are able to generate self-organizing optic cups when cultured under three-dimensional (3D) minimal culture conditions (Eiraku et al., 2011; Nakano et al., 2012). Most importantly, mESC- and hESC-derived optic cups can generate fully laminated neural retina containing all classes of retinal cells including the light sensitive photoreceptors, following the normal sequence of retinal development (Fig. 2). A surprising finding of this study was that optic cups formed independently of any interaction with neuroepithelial cells, surface ectoderm or mesenchymal tissues that ordinarily surround them in the developing embryo, challenging our traditional understanding of this developmental process. Importantly, murine rod precursors arising from the 3D differentiation system described above have the ability to integrate within the degenerate retina in adult mice and mature into photoreceptors showing outer segment formation (Gonzalez-Cordero et al., 2013), while no outer segments were observed in optic cups derived from hESCs.

Human transplantation studies have yet to be attempted with hESC and hiPSC 3D-derived photoreceptors; nevertheless, the above findings highlight an important facet of ESC and iPSC biology that is essential for the field of retinal regeneration: a latent intrinsic ability to self-organize giving rise to a multilayered neural retina which can be exploited for drug discovery purposes, disease modeling, and retinal regeneration. Such *in vitro* grown retina has profound potential for determining the molecular and inductive interactions that are essential for eye development which have not yet been elucidated from embryological studies due to the scarcity of human embryonic material. Furthermore, the ability to produce laminated neural retina containing functional photoreceptors with fully formed outer segments is invaluable for producing *in vitro* models of retinal disease that are sufficiently close to *in situ* retina in order to obtain clinically useful results. The current cost of bringing a new drug to the market has been evaluated in the range of 4 to 11 billion USD. This has been associated with a high failure rate due to the lack of appropriate biological models that reflect patient populations. The reproducible generation of fully functional neural retina from a large number of hESC and hiPSC lines offers an amazing prospect for drug discovery and disease modeling using hiPSCs generated from patient tissues. The generation of photoreceptor precursors within a 3D *in vitro* microenvironment which closely resembles that of the developing human retina may also be our best chance of producing photoreceptor precursors that have received the relevant "priming" signals during their development for them to act in an appropriate functional manner following transplantation in humans. It is also possible that transplantation of combined RPE/retinal sheets derived from pluripotent stem cells may be a successful strategy as carried out in pilot studies with human fetal tissue (Radtke et al., 2008).

Given the incredible promise of pluripotent stem cell differentiation, it is not surprising that clinical safety studies focused on the more straightforward challenge of transplanting hESC and hiPSC-derived RPE have already started (Table 1). The first trial was approved by the U.S. Food and Drugs Administration in January 2011 to enable Advanced Cell Technology (ACT) to perform a phase I/II multicenter clinical trial to treat dry AMD patients with RPE cells derived from hESCs. Japan has already approved the second clinical trial, scheduled to start in September 2013, and is intended to treat six patients with wet AMD using autologous RPE cells obtained from iPSCs. Three additional trials (Table 1) are also imminent in the United Kingdom, United States, and Korea. Although the long-term outcome of these trials is eagerly awaited, early results from the ACT study have underlined the safety and tolerability of these cells for clinical trials and have set the scene for pioneering new therapies for retinal disease (Schwartz et al., 2012).

What does the future hold?

It is clear that the generation of laboratory grown synthetic retina from hESCs and hiPSCs provides one of the best tools to date for modeling human retinal disease, large-scale drug screening, and disease reversion by transplantation of patient-specific photoreceptors and/or RPE following the correction of disease-linked gene mutations. To realize these goals, we need to optimize current differentiation protocols to achieve the *efficient and reproducible* generation

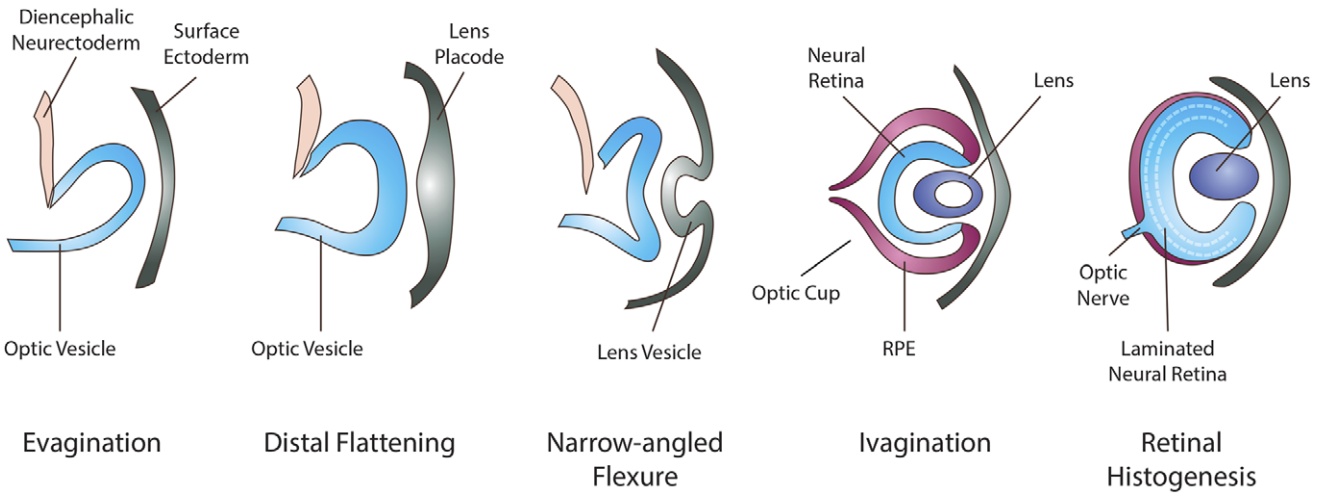


Fig. 2. Schematic representation of retinal ontogenesis.

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of synthetic retina from a large number of hESC and hiPSC lines *in defined media conditions and within time periods* that are amenable to human cell replacement therapies. Current hESC-based studies suggest that it takes up to 126 days to generate a fully laminated neural retina (Nakano et al., 2012). Moreover, culture media commonly include fetal bovine serum (FBS) which is of animal origin and can show batch-to-batch variability and hence is not suitable for clinical applications (Nakano et al., 2012). Under these conditions, around 58% of hESC-derived aggregates contain optic-like vesicles. However, the optic vesicles that undergo further differentiation to stratified neural retina contain photoreceptors lacking outer segments and therefore unable to respond to light. In conclusion, there remains clear scope for improvement and below we have highlighted several methods that may enhance our ability to generate functional photoreceptors:

The provision of a microenvironment which allows endogenous signaling to guide retinal formation

Most cell culture–based studies tend to rely on the application of fetal bovine serum or similar substituents (for example knock-out serum [KSR]) to achieve either cell expansion or differentiation toward desired lineages. This has proven to be counterproductive in protocols designed to generate synthetic retina, as knock-out serum caudalises hESC-derived neural progenitors, contributing to low yields of ESC-derived optic cups which ordinarily emerge from anterior forebrain (diencephalon) during embryonic development (Eiraku et al., 2011; Nakano et al., 2012). Indeed, lowering the knock-out serum concentration results in greater efficiency of optic cup formation from both hESCs and mESCs (Eiraku et al., 2011; Nakano et al., 2012). Our work and that of others have shown that this is due to the ability of differentiating hESCs to endogenously upregulate the expression of a range of factors important for retinal cell type specification (Mellough et al., 2012). For example, embryoid bodies obtained from hESCs are able to upregulate the expression of *EGF*, *DKK1*, *NGF*, *NODAL*, and *SHH* when allowed to differentiate in the absence of any added morphogens or growth factors. This suggests that, in the absence of external cues, a proportion of hESCs follow an intrinsic neural and retinal default differentiation pathway. However, when these conditions are maintained long term, we have also noticed a delay in the onset of expression of mature photoreceptor markers, which can be rescued by the addition of two culture supplements, N2 and B27, at specific stages of the differentiation process (Mellough et al., 2012). Combining these two nutrients with matrigel, a gelatinous protein mixture mimicking the complex extracellular environment under bioreactor culture conditions (allowing more efficient exchange of gas and nutrients), has facilitated the generation of cerebral organoids containing an immature neural retina within 3 weeks of culture, although the efficiency, reproducibility, and functional maturity of this system remain to be investigated further (Lancaster et al., 2013).

Modulation of extracellular signaling

The retina is derived from RPCs, a partially restricted neuroepithelial cell population that undergoes rapid and dramatic expansion in size by proliferation and differentiation to give rise to retinal neurons in a temporally ordered sequential fashion (Swaroop et al., 2010). Photoreceptors have a complex, highly unique and specific phenotype, many aspects of which arise from their interaction with other retinal cell types as well as with the vitreous and outer limiting membrane of the retina. In the last twenty years, several studies have demonstrated that the competence of RPCs to generate each of the specific retinal cell types can be altered by manipulating both intrinsic and extrinsic factors (Swaroop et al., 2010). While these studies have been performed in various animal models (chick, frog, mouse), there is a general consensus that six major families of signaling molecules, namely Shh, transforming growth factor beta (TGFβ), BMP, Wnt, fibroblast growth factor (FGF), Notch, and IGF-1 govern key events during retinogenesis. The process begins with the establishment of bilateral apically concave optic vesicles and their subsequent invagination to form the optic cup, a bilayered apically convex structure, determining cell fate commitment to RPE (the outer layer of the optic cup) or neural retina (the inner layer), influencing RPC proliferation, exit from cell cycle and the decision to become rod/cone photoreceptors, retinal ganglion cells (RGCs), Muller cells, and each of the retinal interneurons. This developmental knowledge has been incorporated into hESC differentiation protocols (Table 3). For example, the application of Shh agonists and Notch inhibitor to cultures during the latter stages of differentiation (Nakano et al., 2012; Eiraku et al., 2011; Lund et al., 2006) enhances the generation of photoreceptors, as expected from developmental studies in animal models (Table 3). Similarly, the addition of IGF-1 to the culture media enhances the generation of RPCs from hESCs (Lamba et al., 2006), in accord with the multiple roles played by IGF-1 during eye development (Table 3). While application of some of those factors has already been tested in the most recent 3D hESC differentiation protocol (Eiraku et al., 2011), these experiments were undertaken in the presence of FBS which, by its undefined composition and containment of multiple signaling molecules, may hide or mask the true effects of the signaling molecules being tested. It is imperative now to apply those extracellular signaling molecules to define minimal media at particular stages of differentiation to assess their impact on optic cup emergence, neural retina and RPE formation, emergence of mature photoreceptors, and formation of fully formed neural retina. Of equal interest is to determine whether manipulation of the hESC differentiation cultures with FGFs (for example FGF8 or 9) upon the emergence of the bilayered optic cup is able to alter the balance between neural retina and RPE formation. This is important for designing directed differentiation strategies for the production of desired cell types for use in cell replacement therapies or drug discovery studies. From a scientific and biological point of view, it is also of tremendous interest to investigate the expression of these signaling molecules, their receptors and downstream effectors in hESC/hiPSC-derived synthetic retina and to compare and contrast those to native developing human retina. This will provide the answer to whether we are able to replicate the formation of retinal cell types using pluripotent stem cells as it happens *in vivo*.

Modulation of oxygen concentration

A number of studies have revealed that oxygen (O_2) is a potent regulator of stem cell maintenance and differentiation, thereby controlling the provision of O_2 to the ESC or iPSC differentiation cultures could provide a powerful tool for enhancing differentiation regimes (Forristal et al., 2010; Mondragon-Teran et al., 2011*a*). Oxygen tension in the uterus of mammalian species ranges from 1.5 to 8.7%. In humans, the mean O_2 tension at the uterine interface during weeks 7–10 of gestation is 2.4% and after 11 weeks rises to 7.8% as a result of maternal blood flow to the fetus (Rodesch et al., 1992; Fischer & Bavister, 1993). Although the O_2 tension of the human diencephalon from which the optic cup emerges has never

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Table 3. *Summary of key signaling molecules involved in retinal ontogenesis*

Signaling molecule	Role in retinal ontogenesis	Role in hESC/iPSC differentiation	References
Hedgehog family	**Sonic hedgehog (Shh):** Important for formation of separate optic cups, establishing the boundary between ventral and dorsal optic primordium and lamination of neural retina. Shh is produced by RPE and when overexpressed leads to conversion of ventral RPE and optic stalk to neural retina and an enhancement of photoreceptor cell proliferation and differentiation. Inhibition of Shh signaling during the peak of RGC genesis enhances the generation of RGCs.	Addition of Shh agonist to later stages of hESC differentiation augments retinal differentiation	(Stenkamp & Frey, 2003; Eiraku et al., 2011; Mellough et al., 2012)
TGFβ/BMP family	**Activin A**: Important for induction and maintenance of RPE markers, encourages exit of photoreceptor progenitors from cell cycle, and promotes differentiation of photoreceptor precursors to rods.	Addition of activin A enhances formation of RPE	(Davis et al., 2000)
	BMP4: Together with Shh, responsible for dorsoventral patterning of optic cup.	Inhibition of BMP signaling enhances first stages of hESC differentiation by promoting anterior neural specification	(Sakuta et al., 2001; Osakada et al., 2008; Hirami et al., 2009; Mellough et al., 2012)
Wnt family	**Wnt 13**: Expressed in the ciliary margin and implicated in retinal progenitor proliferation and differentiation.	Inhibition of Wnt signaling enhances first stages of hESC differentiation by promoting anterior neural specification	(Kubo et al., 2003; Osakada et al., 2008; Hirami et al., 2009; Mellough et al., 2012)
FGF family	**FGF9**: Transient expression of FGF9 in RPE causes its conversion to neural retina.		(Pittack et al., 1997; Patel & McFarlane, 2000; Vogel-Höpker et al., 2000; Zhao et al., 2001; Kubo et al., 2003; Catalani et al., 2009)
	FGF8: Transient expression prior to contact of the optic vesicle with surface ectoderm causes optic vesicle regression. Later expression in RPE causes its conversion to neural retina.		
	FGF1: Transient expression of FGF1 in RPE causes its conversion to neural retina. FGF1 is expressed at high levels in peripheral retina and its overexpression accelerates the wave of RGC differentiation, suggestive of an important role in RGC maturation.		
	FGF2: Expressed in head ectoderm at the very early stages of optic cup formation and later on in the neural retina. Its expression in RPE causes conversion of RPE to neural retina. Overexpression of FGF2 enhances the generation of rod photoreceptors at the expense of cones and promotes RGC generation at the expense of Muller cells.		
Retinoic acid (RA)	**RA** is produced at high concentrations in developing retina as early as optic cup stage formation. Expression shifts to RPE at later stages. Addition of RA to culture media promotes differentiation of photoreceptor precursors to rods.		(Kelley et al., 1994)
Taurine	Addition of taurine to the media promotes differentiation of photoreceptor precursors to rods.		(Lombardini, 1991)
Notch	Disruption of canonical Notch signaling in early retinogenesis results in accelerated RPC differentiation/exit from the cell cycle and disruption of retinal lamination. Also Notch signaling governs the determination of RGCs, Muller cells, and differentiation of RPCs to rod or cone photoreceptors.	Inhibition of Notch signaling facilitates differentiation to photoreceptors.	(Osakada et al., 2008; Riesenberg et al., 2009; Zheng et al., 2009; Eiraku et al., 2011; Nakano et al., 2012; Mizeracka et al., 2013)

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Table 3. Continued.

Signaling molecule	Role in retinal ontogenesis	Role in hESC/iPSC differentiation	References
IGF	Injection of IGF-1 mRNAs in *Xenopus* embryos leads to formation of ectopic eyes containing multi-layered neural retina, RPE and sometimes lens. Consistent with a role in retinal development, expression of the IGF-1 receptor is observed from the very early stages of optic cup formation in human embryos, later becoming restricted to the lens and RPE at 6 weeks of development. As retinal maturation proceeds, IGF-1 expression is also observed in post-mitotic retinal precursors which are in the process of differentiating into cones and in the inner segments of photoreceptors promoting cone and rod survival. IGF-1 expression is also observed in rod outer segments and has the ability to phosphorylate rod transducin, indicating that IGF signaling may be involved in light transduction. Mice lacking insulin receptor substrate 2, an essential component of the IGF-1 signaling cascade show 50% loss of photoreceptors by two weeks of age and almost complete loss of photoreceptors by 16 months of age. Overexpression of IGF-1 results in significant improvements in engraftment of postmitotic rod precursors cells in adult retina.	Addition of IGF-1 to differentiating hESC increases the number of emerging retinal progenitor cells.	(Coppola et al., 2009; Rodriguez-de la Rosa et al., 2012; Pera et al., 2001; Yi et al., 2005; West et al., 2012; Forristal et al., 2010; Lund et al., 2006)

been directly measured, there is an understanding from work done in other species that a low O_2 tension (between 0.8% and 4%) is prevalent across different locations of the developing brain. In accordance with these findings, it has been shown that lowering oxygen levels to 2% during the differentiation of murine and hESC results in a significant increase in the expression of key retinal markers and the yield of photoreceptors (Garita-Hernández et al., 2013; Bae et al., 2012). Although the exact mechanisms by which hypoxia improves differentiation to retinal photoreceptors are not yet known, it is envisaged that this is orchestrated by stabilization of hypoxia-inducible factor 1-alpha (Hif1α) and subsequent activation of VEGF, both known to have neuroprotective and proliferation inducing abilities in RPCs (Grimm et al., 2002). Mammalian embryos are however exposed to increasing O_2 as development proceeds, and in the very early stages of postnatal development (postnatal day 10–20) hypoxia has counterproductive effects resulting in photoreceptor death (Maslim et al., 1997; Mervin & Stone, 2002); hence, increasing the O_2 concentration in a stepwise fashion as suggested by Mondragon-Teran et al., 2011*b* may yield improved differentiation results. This, combined with controlled and reproducible generation of floating 3D structures using custom-made tissue culture wells (for example aggrewells) subsequently expanded in bioreactors, which enable more efficient oxygen distribution within differentiating cultures, could provide the support that is needed for more efficient generation of synthetic retinae from pluripotent stem cells.

Modulation of the extracellular matrix

The extracellular matrix (ECM) plays an important role in regulating stem cell proliferation and providing inductive cues for differentiation (Kazanis et al., 2010; Keung et al., 2011; Keung et al., 2012). ECM can immobilize secreted proteins and alter their biochemical activities so that they are able to exert highly specific effects upon the cells in their vicinity. Moreover, changes in ECM characteristics can influence signal transduction systems and regulate the movement and migration of stem cells; hence, the ECM is a critical component in establishing and maintaining the stem cell microenvironment. Both aspects have been shown to be important for pluripotent stem cell differentiation, for example the presence of soft ECM promotes the early neural differentiation of iPSCs (Keung et al., 2012; Boucherie et al., 2013), while addition of Matrigel to the media enhances human and mESC self-organization into optic cup-like structures under three-dimensional culture conditions (Eiraku et al., 2011; Nakano et al., 2012). Together these data suggest that the manipulation of ECM characteristics is a promising method that could influence pluripotent stem cell differentiation toward synthetic retina. Defining the optimal ECM components that are physiologically relevant during human retinal development and which may enhance the hESC/iPSC differentiation system to synthetic retina will therefore be of great importance. Multiple lines of published evidence suggest that laminins and their receptors are key candidates that deserve attention when designing new differentiation regimes. Laminins are a family of heterotrimeric glycoproteins, each composed of an α, β, and γ chain that combine to form at least 15 different laminin isoforms. Several members of this family (α3, α4, α5, β2, β3, γ2, and γ3 chains likely to organize into Laminin 5, 14, and 15) are expressed in the interphotoreceptor matrix (IPM) during murine retinal development, prior to and during photoreceptor differentiation (Libby et al., 2000). Single and double knockdown studies of β2 and β2/γ3 chains have respectively shown their importance in

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the maintenance of rod photoreceptors (Hunter et al., 1992; Libby et al., 1999), formation of synaptic ribbons and photoreceptor outer segments, the integrity of the inner limiting membrane, Müller cell attachment and retinal organization (Pinzón-Duarte et al., 2010). Furthermore, disruption of integrin α6 that functions together with integrin β1 as a major laminin receptor leads to abnormalities in retinal laminar organization in mice (Georges-Labouesse et al., 1998), suggesting that laminins and their receptors are key players in the development of several retinal cell types as well as in the maintenance of retinal cyto-architecture. Indeed, the replacement of matrigel with laminin during the differentiation of murine ESCs under 3D conditions allows the successful generation of optic cups and their further differentiation to a fully stratified neural retina, although this remains to be tested in human tissue (Eiraku et al., 2011). Furthermore, disruption of integrin signaling results in the inhibition of apical nuclear deviation, giving rise to an unusually thin hESC-derived neural retina that fails to evert suggesting that, at least in the human scenario, ECM driven integrin signaling is critical for the formation of the neural retina (Nakano et al., 2012).

Recapitulation of the interphotoreceptor matrix microenvironment

The IPM surrounds the photoreceptors inner and outer segments and plays an important role in the trafficking of retinoids and metabolites, maintenance of the photoreceptor-specific microenvironment, photoreceptor alignment and cellular interactions between the outer segments and RPE (Hollyfield, 1999). It is tempting to speculate that recreation of the embryonic IPM microenvironment during hESC and hiPSC differentiation may provide the critical microenvironmental cues needed for the formation of photoreceptor outer segments *in vitro*. A major component of the IPM in human adult retina is hyaluronic acid (HA), a large non-sulfated linear polysaccharide of (1-β-4)D-glucuronic acid and (1-β-3)*N*-acetyl-D-glucosamine, which is able to bind a number of secreted proteins of IPM (for example SPACR and SPACRCAN) and also forms a scaffold that fills the IPM (Hollyfield, 1999; Keenan et al., 2012). Formation of this scaffold is enabled by link proteins (for example HAPLN1, shown to be present in the IPM), which form a ternary complex with HA and proteoglycans (for example aggrecan, also expressed in the IPM) (Keenan et al., 2012). Whether this is the case in developing human retina is unknown, but an important aspect of future investigation. Identification of proteoglycans that are expressed during distinct stages of human retinal ontogenesis is likely to greatly facilitate improvements in existing differentiation regimes. With current improvements in tissue engineering and polymer scaffolds, one can easily envisage the encapsulation of hESC and hiPSC into HA hydrogels. The advantage of this system is that other ECM components and specific growth factors can be added and immobilized to create a more efficient environment for hESC and hiPSC differentiation toward synthetic retinas *in vitro*. Recent findings indicate that HA hydrogels can be successfully used to deliver donor cells to the retina and help minimize cell aggregation and degradation in the first week following transplantation (Gerecht et al., 2007). Optimization of 3D differentiation regimes using HA hydrogels in longer term may not only be beneficial for improving *in vitro* differentiation, but also for delivering the hESC- and hiPSC-derived retinal structures *in vivo*.

The power of iPSC-based approach for disease modeling, drug discovery, and cellular therapies

In retinal disease with monogenic inheritance where the gene defect is known, genetically modified animals produced by targeting specific gene functions have been the mainstay of modeling and investigating disease mechanisms. This is not the case for complex retinal disease (such as AMD) or inherited gene disorders where the gene defect is unknown, for which no truly representative disease models have yet been created. In addition, several key differences exist between animal models and humans in terms of lifespan, tissue composition, anatomy and physiology. Mice typically live for 1–2 years and have no fovea (the centrally positioned cone-enriched region of the retina that is essential for fine visual acuity in humans). Furthermore, it is known that even single gene defect disorders can be affected by non-pathological variations in a large number of other genes in addition to as yet poorly understood epigenetic differences. This is particularly important in complex polygenic diseases (such as AMD) where a large number of identified genetic associations are thought to be disease modifying rather than disease causing, as well as single gene defects with varying penetrance (such as RP caused by mutations in the splicing factor *PRPF31*). In the same way that studying disease mechanisms is limited by representative tissue, the assessment of any therapeutic effect of novel agents and understanding variable pharmacodynamics is limited by the lack of suitable disease models. Together these studies highlight an imminent need for the generation of human disease-specific models, which can be used for drug screening and investigating disease pathology. Postmortem eyes can be used to investigate the pathophysiology of disease, but have limited availability, and such samples often represent end stage disease that has been altered by secondary disease processes rather than evolving disease. Although some complex diseases such as AMD are relatively common, the acquisition of tissue samples from all disease variants would be difficult and time consuming. In addition, studies that focus on the correlation between cellular function and clinical phenotype/molecular genotype are difficult to perform with high accuracy using postmortem material.

The advent of iPSC technology and the relative ease with which patient somatic cells can be reprogrammed to pluripotency have opened new and exciting avenues for *in vitro* disease modeling. hiPSCs can self-renew indefinitely in culture, endogenously express pluripotency genes at normal levels, and bear the genetic profile of the patient, thus increasing the likelihood of recapitulating important disease mechanisms. In the case of retina, this is particularly beneficial, since retinal tissue is not amenable to routine tissue biopsy, and methods already exist to coax hiPSCs toward photoreceptors and RPE cells (refer to Table 2). The key question is whether hiPSC-based modeling can provide a platform for all retinal degenerative diseases or be limited to some forms only. This is very dependent on disease complexity, the retinal cell type being affected and the stage of disease onset; for example, age-related diseases may be harder to model. It is unlikely that many diseases affecting multiple cell types and organs and triggered or influenced by environmental factors that are difficult to control (e.g. lifestyle or diet) can be easily modeled with the iPSC approach. However, RPE-based disorders are ideal for this type of modeling, given the ease of RPE generation from hiPSCs using simple protocols and the ability of hiPSC-RPE cells to display key morphological, physiological, and functional features akin to primary human RPE. Furthermore, proposed environmental effects can be mimicked. Indeed, Singh et al. have used this approach to model Best Vitelline

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Macular Dystrophy (BVMD), an autosomal dominant disorder with highly variable age of onset, which often begins in childhood or adolescence and is caused by over 100 mutations in the BEST1 gene, manifesting in the appearance of a yellow "egg yolk" lesion in the subretinal space (Singh et al., 2013). Although BVMD is known to arise from malfunction of the RPE leading to secondary photoreceptor degeneration, there are no clear conclusions as to how mutations in *BEST1* cause the RPE to malfunction. Derivation of BVMD patient iPSCs and their differentiation into RPE indicates that RPE derived from BVMD patients and unaffected controls show similar molecular and physical characteristics under steady state conditions. Upon exposure to physiological stress (such as long-term feeding with photoreceptor outer segments or the addition of exogenous ATP to the culture media), reduced fluid transport in patient-derived RPE was observed, thus recapitulating the clinical features of BVMD. Further investigation into the role of BEST1 using hiPSC-derived RPE also revealed an important role for BEST1 in endoplasmic reticulum-mediated calcium transients, thus providing for the first time a conclusive mechanism that links BEST1 function to BVMD. In a similar study, this time using photoreceptors generated from patients with sporadic RP, Tucker et al. were able to uncover new insights into the consequences of *Alu* insertion discovered by exome sequencing studies (Tucker et al., 2011). Using elegant molecular biology analysis, the authors were able to show that Alu insertions into the exon 9 of a patient's *MAK* gene prevented the expression of a specific retinal isoform which is expressed in adult human retina, thus uncovering new mechanisms that link the disease causing mutation to the transcriptome of photoreceptor cells (Tucker et al., 2011). In addition to uncovering new insights for RP modeling, this study makes a compelling case for the usefulness of an iPSC modeling approach that is able to provide access to patient-specific photoreceptor cells that can be used to address explicit questions about the function and expression of particular candidate genes in retinal cells. hESCs can also be used to model retinal disease caused by single gene mutations, as these can now be easily introduced in the genomes of cells using engineered nucleases (such as ZFNs, TALENs, or CRISPR/CAS9 based methods). However, diseases that are influenced by extrinsic or epigenetic factors, or show varying penetrance such as PRPF31 RP cannot be accurately modeled this way, highlighting once more the value of patient-specific hiPSC-based modeling.

In addition to gaining insights into disease onset and pathology, hiPSC-derived retinal cells from both patients and unaffected controls can be used as a platform to discover new drugs and test the pharmacological effects of existing drugs. To date there have been only two studies investigating the effects of existing drugs on iPSC-derived retinal cells. In the first study, Jin et al. generated iPSCs from patients with RP caused by mutations in *RP1*, *PRPH2*, *RHO*, and *RP9* and observed a significant degeneration of iPSC-derived rod photoreceptors under *in vitro* conditions between days 120 and 150 of differentiation. Treatment with α-tocopherol (acting as an antioxidant) rescued rod photoreceptor death in cell lines from patients with *RP9* mutations, but not in experiments using lines harboring other forms of RP causing mutations, suggesting that this approach can be used to quickly screen new drugs and narrow down the number of candidates that go forward to clinical trials to treat particular forms of retinal disease, making the process safer and more effective (Jin et al., 2011). In a second study, Meyer et al. were able to derive iPSCs from a patient with gyrate atrophy, a rare autosomal recessive retinal disorder that primarily affects the RPE, leading to secondary photoreceptor loss and degeneration (Meyer et al., 2011). Supplementation of culture media with vitamin B6 resulted in the restoration of ornithine transferase activity in RPE cells derived from this patient, but not the fibroblasts used for hiPSC derivation, underscoring once more the necessity to screen drugs on the affected retinal cell type.

To date, neither 2D nor 3D culture approaches have resulted in the generation of mature photoreceptors bearing outer segments, which are capable of transducing light *in vitro*. This may pose some limitations when trying to understand the functional consequences of gene mutations using patient-derived retinal cells. The possibility of generating fully laminated neural retina under 3D culture conditions (Eiraku et al., 2011; Nakano et al., 2012) has however opened new horizons for investigations of this nature, but still relies on some fine tuning of the retinal production process for it to be of greatest use. Nonetheless, 3D generated neural retina will be particularly important for investigating the genesis of specific photoreceptor types (rods *vs.* cones) in order to study congenital disorders such as enhanced S-cone syndrome, which manifests as a gain in the number and function of S cones (short wave length, blue cones), night blindness and varying degrees of L (long red) and M (middle green)-cone vision due to mutations in *NR2E3* gene. It is speculated that this disorder is caused by a fault in the process determining photoreceptor identity, but how this develops in humans remains unclear. *In vitro* 3D generated neural retina presents an unprecedented opportunity to address these questions and to assess photoreceptor production during human development (Haider et al., 2000).

Recent developments in targeted genetic engineering through the use of ZFN, TALENs, and CRISPR/CAS9 technologies have made the correction of mutations at endogenous loci in hESC and hiPSC possible with much greater ease and efficiency. Although some questions still remain regarding potential harmful consequences of off-target effects in the genome, the prospect of combining stem cell and gene therapy holds great promise for restorative therapies in the eye. To date, there has been only one successful report of the functional correction of a retinal disease causing mutation in patient-specific hiPSC using bacterial artificial chromosome mediated homologous recombination (Meyer et al., 2011), and no doubt additional examples will emerge as the technology moves forward. The correction of a gene defect in hiPSCs coupled with 3D differentiation would not only provide a powerful tool with which to generate fully functional patient-specific retinal cells, but also an appropriate control that encompasses the genetic background and environmental influences when investigating patient-specific hiPSC for disease insights and drug testing.

The first proof of principle studies highlighted in this review and encompassing literature has clearly demonstrated the utility of hiPSC for disease modeling, drug screening, and future cell replacement therapies. However, one needs to be aware of a number of issues related to reprogramming strategies (genetic and epigenetic differences between hiPSC clones derived from the same patient and between patients, incomplete reprogramming, imprinting, repeat instability, copy number variations, and mutations that may arise during reprogramming etc.), banking of hiPSC (availability of GMP compatible hiPSC derivation protocols, scalability, numbers of hiPSC lines that need to be banked in each country), safety (lack of tumor formation upon transplantation), development of robust differentiation protocols for generating mature photoreceptors (scalability, GMP compliance, timing), and identification of disease relevant and interesting phenotypes that can benefit from the benefits that the hiPSC system offers. All these issues have been well addressed in a recent review to which the reader is encouraged to refer (Wright et al., 2014) and for this reason will not be expanded herein.

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Conclusions and future directions

While the progress of pluripotent stem cells for retinal disease modeling and treatment has surpassed most expectations, a number of hurdles in the clinical, biological, and regulatory field need to be addressed before the promise of lab-made synthetic retina becomes a reality. At the moment, it is not clear whether gene corrected patient-specific iPSC will make its way to the clinic and pave the path toward personalized medicine; or will generic retinal cells created from carefully selected hiPSC and hESC banks on the basis of homozygosity for HLA-A, B, and DR ride the fast track to the clinic? A series of careful considerations must be made before these decisions can be reached and will of course also be influenced by cost, the time it takes to prepare the required retinal cells before individual treatments can commence, ease of distribution to the clinics, and legislation that governs health and safety across countries. As is often the case, one approach is unlikely to provide an all-encompassing solution; instead we will likely witness the tailored application of both hESCs and hiPSCs across various aspects of disease modeling, drug screening, drug discovery, and cell-based replacement therapy. Indeed, ongoing clinical safety trials using hESC-derived RPE in the United States and hiPSC-derived RPE in Japan are an important test of current approaches and provide an excellent indication of how far and fast we have come toward developing new therapies for blinding diseases from the first derivation of hESC in 1998 and hiPSC in 2007. If we can achieve such progress in just 15 years, no doubt the next 15 years will be an extremely exciting period for visual research and is anticipated to reveal new knowledge about disease mechanisms and the advent of therapies to restore vision in patients affected by currently untreatable retinal disease.

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