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SPECIAL ISSUE

Strategies for Restoring Sight in Retinal Dystrophies

REVIEW ARTICLE

Photoreceptor replacement therapy: Challenges presented by the diseased recipient retinal environment

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Abstract

Vision loss caused by the death of photoreceptors is the leading cause of irreversible blindness in the developed world. Rapid advances in stem cell biology and techniques in cell transplantation have made photoreceptor replacement by transplantation a very plausible therapeutic strategy. These advances include the demonstration of restoration of vision following photoreceptor transplantation and the generation of transplantable populations of donor cells from stem cells. In this review, we present a brief overview of the recent progress in photoreceptor transplantation. We then consider in more detail some of the challenges presented by the degenerating retinal environment that must play host to these transplanted cells, how these may influence transplanted photoreceptor cell integration and survival, and some of the progress in developing strategies to circumnavigate these issues.

Keywords: Transplantation, Photoreceptor, Degeneration, Gliosis, Stem cells

Introduction

Retinal degenerations leading to the loss of photoreceptors are a major cause of untreatable blindness in the developed world. Inherited retinal dystrophies, typically grouped under the umbrella of retinitis pigmentosa (RP), affect one in 3000 of the population and age-related macular degeneration (AMD) affects one in 10 people over 60 years, a figure that is rising with our increasingly aging population (Minassian et al., 2011; Owen et al., 2012). Currently, there are few effective treatments. None are able to both replace lost photoreceptor cells and restore visual function and there is thus a clear need for new therapeutic approaches.

There has been considerable interest around the potential for retinal repair by cell replacement strategies. Indeed, the eye is considered by many to represent one of the most feasible targets for such novel therapeutic approaches. It is a highly tractable system for the delivery of treatments *via* existing surgical techniques and the scene for the application of pioneering therapies for the treatment of retinal disease has already been set with the initiation of the first gene therapy trials for inherited RP (Bainbridge et al., 2008; Maguire et al., 2008). The success of gene therapy relies on the delivery of new functional genes to cells that lack such genes and is therefore entirely dependent upon endogenous cell survival. In cases where the degenerative process has already led to cell death or in those conditions that are not amenable to gene therapy approaches, cell replacement may offer a complementary approach.

The prerequisites for successful neural transplantation are many and complicated, including successful migration and integration of the donor cell into the target tissue, correct differentiation into the appropriate cell type, synaptic connectivity with downstream targets and restoration of function, and long-term survival, all set within the context of a retinal environment undergoing degeneration. Despite this enormous challenge, the photoreceptor is a surprisingly amenable target for cell replacement strategies, being an afferent neuron with a single synaptic connection and one for which the anatomical location of the cell largely determines its spatial contribution to the retinotopic map (see Fishman, 1997). Together, these factors make the rewiring of a transplanted photoreceptor cell easier than the majority of other neuronal cell types and the outer retina perhaps one of the most permissive regions of the adult CNS in which transplanted neurons could make functional connections.

Recent progress in photoreceptor transplantation

Transplantation with the aim of replacing lost photoreceptors has been approached using a number of different strategies, including the transplantation of whole retinal sheets (Turner et al., 1988; Ghosh et al., 1999, 2004; Seiler et al., 2008) and microaggregates of neural retina and of cell suspensions of stem cells and progenitor cells. A full review of these strategies, the specific cell types used

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and their outcomes is beyond the scope of this review and we comment briefly only on the transplantation of dissociated cells (but see West et al., 2009; Pearson, 2014). Dissociated cells from non-retinal sources of donor cells, typically hippocampal-derived neural progenitors, frequently fail to differentiate into mature retinal phenotypes, particularly photoreceptors (Young et al., 2000; Sam et al., 2006; Mellough et al., 2007), after transplantation. Given this apparent restriction in fate, a number of groups have examined the transplantation potential of progenitor cells isolated from embryonic retinas, which possess the potential to differentiate into retinal neurons. In the majority of studies, these have been expanded in culture prior to transplantation and, depending upon the culture conditions used during expansion, have variously shown these cells to survive and differentiate into glial cells (Yang et al., 2002) and/or cells expressing retinal-specific markers including some specific for photoreceptors (Qiu et al., 2005). However, they typically fail to correctly migrate into the laminar structure of the neural retina and thus integrate into the recipient visual system. Moreover, the morphology of these integrated cells did not resemble mature photoreceptors. Sakaguchi, Young and colleagues demonstrated that transplantation was more effective when transplanting cells into immature recipients. Cultured murine neural progenitor cells transplanted into the eyes of neonatal Brazilian Opossums, which retain an immature environment for many days after birth, migrated into the recipient retina, although integrated cells were typically located in the inner retina, and not found in the outer nuclear layer (ONL), where photoreceptors normally reside (Sakaguchi et al., 2004, 2005). By transplanting in the subretinal space a mixed population of retinal cells that were isolated from the developing retina at a stage when rod photoreceptors are immature, we, and others, have shown that the non-neurogenic, adult retinal environment is able to incorporate and support the maturation of new photoreceptors (Kwan et al., 1999; MacLaren et al., 2006; Bartsch et al., 2008). Importantly, MacLaren et al. (2006) demonstrated that successful transplantation occurs when the donor cells are at an appropriate stage in development at the time of transplantation. By using a genetic marker, Nrl, which is expressed in immature photoreceptors shortly after terminal mitosis (Akimoto et al., 2006), we were able to show that these results are achieved by using photoreceptor precursor cells—cells that are specified to differentiate into photoreceptors—but not progenitor cells or photoreceptors at other stages of development (MacLaren et al., 2006) as confirmed by others (Bartsch et al., 2008; Eberle et al., 2011; Lakowski et al., 2011). When transplanted, these rod photoreceptor precursors migrate to the ONL, form new synaptic connections with inner retinal neurons (Pearson et al., 2012), mature in a manner similar to normal rods (Warre-Cornish et al., 2013), and elaborate new outer segments (Eberle et al., 2012; Pearson et al., 2012; Warre-Cornish et al., 2013). Importantly, these new rods are capable of restoring rod-mediated vision in some models of retinal degeneration (Pearson et al., 2012; Barber et al., 2013). Others have suggested that it is possible to transplant adult photoreceptors, taken from a mixed population of neural retinal cells (Gust & Reh, 2011), but the efficiency of integration of these fully mature cells is very low compared with photoreceptor precursor cells, even when factoring in cell viability (Lakowski et al., 2011; Gonzalez-Cordero et al., 2013; Pearson, unpublished data).

Why retinal progenitor cells expanded and differentiated *in vitro* fail to integrate after transplantation as effectively as photoreceptor precursors isolated from the developing retina, despite expressing markers of mature photoreceptors, is unclear but it presumably reflects an inability to correctly complete the developmental programme as far as the precursor stage *in vitro*, despite the expression of some postmitotic markers. A detailed analysis of the development of dissociated retinal progenitor cells expanded in culture is still required, but work using embryonic stem (ES) cells and 3D *versus* 2D culture systems (see below and Gonzalez-Cordero et al., 2013) demonstrates that it is possible to achieve only a partial, and incomplete, differentiation with the latter. Similarly, work with retinal progenitors derived from the ciliary epithelium has also reported a failure of these related cells to undergo complete differentiation *in vitro* (Cicero et al., 2009; Gualdoni et al., 2010). A similar scenario may exist for freshly isolated retinal progenitor cells that are then expanded *in vitro*.

It is not possible to translate the findings from these preclinical studies described above directly to a clinical application, since the period encompassing the photoreceptor precursor stage in human development occurs between 10 and 15 weeks of gestation (Narayanan & Wadhwa, 1998), making the acquisition of large numbers of viable photoreceptor precursors from donors very difficult. However, they do define a strategy for photoreceptor cell replacement therapy and overcome a number of obstacles; they show that rod photoreceptor replacement is feasible provided that the correct stage cell is transplanted. Moreover, as this cell type is postmitotic, it avoids the potential hazards associated with tumor formation due to unregulated proliferation of transplanted undifferentiated stem cells. There has been marked progress in recent years in the use of ES cells and induced pluripotential stem (iPS) cells and their directed differentiation toward a photoreceptor fate (Ikeda et al., 2005; Lamba et al., 2006, 2010; Hirami et al., 2009; Osakada et al., 2009; West et al., 2012). Although not the focus of the current review, it is worth noting that by developing the ground-breaking 3D culture systems described by Eiraku et al. (2011), Eiraku and Sasai (2012), and Gonzalez-Cordero et al. (2013) have now generated ES-derived rod precursor cells that are capable of migrating and integrating in a manner virtually indistinguishable from precursors isolated from the developing retina. Such strategies must now be applied to human ES cells and the efficiency of both generation and integration must be improved. Nonetheless, the first trials of hES-derived retinal pigment epithelium (RPE) cell transplants into patients affected by Stargardt disease are underway in the US and the UK (Schwartz et al., 2012). If shown to be safe, such studies should set the stage for future clinical trials of ES-derived photoreceptor cells.

Challenges presented by the degenerating recipient retinal environment

Despite very different etiologies, AMD and most inherited retinal disorders culminate in the same final common pathway, the death of the photoreceptors. As a result, replacement by transplantation is proposed as a broad treatment strategy applicable to all photoreceptor degenerations. The amount of vision restored by photoreceptor transplantation is critically dependent upon the number of donor cells that are correctly incorporated within the remaining recipient neural retina (Pearson et al., 2012; Barber et al., 2013) and the presence of non-integrated donor cells in the subretinal space is detrimental to function (West et al., 2010; Pearson et al., 2012). Efficiency of cell delivery and the related problems of cell survival and rejection have posed a serious barrier to effective transplantation throughout the CNS since the earliest attempts at brain repair. Such issues have been less extensively studied in retinal transplantation, but it is clear that identifying the factors, both methodological and physiological, that impede transplanted cell migration and integration remains a significant challenge to widespread cell replacement in this organ as well.

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Donor cells are typically transplanted into the subretinal space, between the neural retina and the overlying RPE. From here, they migrate out from the cell mass, extending processes toward the neural retina, through the recipient interphotoreceptor matrix (IPM). Having migrated through the IPM, they must cross the outer limiting membrane (OLM), a series of adherens junctions formed between photoreceptors and Müller glia, before migrating through the ONL to an appropriate position (Warre-Cornish et al., 2013). Thus, in addition to the donor cell being at the correct stage in development, their interactions with the recipient retinal environment are also likely to be key in determining the transplantation outcome.

As degeneration progresses, the microenvironment of the retina undergoes a number of significant changes. The loss of photoreceptors causes the cytoarchitecture of the ONL to become disrupted and the OLM can become compromised (e.g., Mehalow et al., 2003). In addition, Müller glial cells undergo reactive gliosis, leading to the formation of a glial scar that can envelope the entire retina at late stages of degeneration (Jones et al., 2003). Not only does this scar form a physical barrier between injured and healthy tissue, it can also act as a reservoir for the accumulation of extracellular matrix (ECM) proteins, including chondroitin sulfate proteoglycans (CSPGs), which are known to be inhibitory to axonal regeneration (Theocharis et al., 2010). Each of these processes is likely to have a significant impact upon the retina, its health and its physiology (Fig. 1). Moreover, such changes are likely to impact upon new therapeutic strategies including gene and cell replacement. Indeed, gliosis has been shown to negatively impact on the efficiency of viral transduction in gene therapy (Calame et al., 2011; Grüter et al., 2005) and the ability of retinal grafts and electronic implants to contact the underlying retina (Zhang et al., 2003). However, until recently there had been no systematic examination of the impact that degeneration within the recipient retinal environment may have on transplantation outcome.

Recently, Pearson and colleagues performed the first comprehensive assessment of rod-photoreceptor transplantation across different models of inherited photoreceptor degeneration (Barber et al., 2013). They found that photoreceptor transplantation was feasible in all six models examined. Importantly, the severity of degeneration was not a determining factor in transplantation outcome and robust donor cell integration was observed even at late stages of degeneration in some models. This is important since, at least initially, such novel therapies would likely be applied to only very advanced cases. However, the etiology associated with a given disease type was shown to impact significantly on both the number and the morphology of integrated rods. For example, rod precursors transplanted into the *Gnat1*$^{-/-}$ model of stationary night blindness integrated in numbers similar to healthy wild-type retina and formed robust outer segments and synaptic connections. Conversely, cells transplanted into the moderately fast degenerating rhodopsin knockout (*Rho*$^{-/-}$), integrated poorly and rarely elaborated outer segments or synapses. In contrast, transplants into the *PDEβ*$^{rd1/rd1}$ model, which degenerates rapidly with little or no ONL remaining by 6 weeks of age, demonstrated surprisingly good integration, with *Nrl*.GFP^{+ve} donor cells found interspersed between the remaining recipient cone photoreceptors, a finding supported by others,

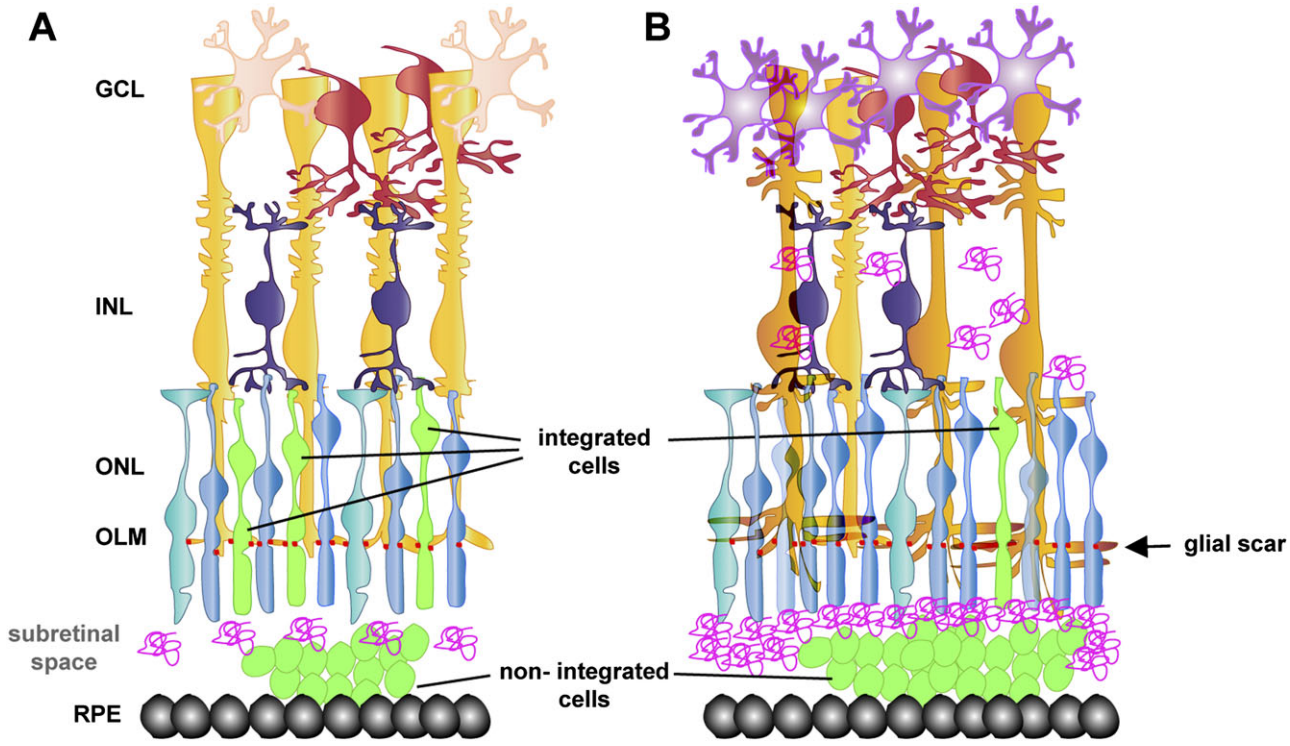


Fig. 1. Environmental factors in the recipient retina that may impede transplanted photoreceptor integration. Donor photoreceptors (green) or other cells typically are transplanted into the subretinal space. In wild-type retinae (**A**), they must migrate through the interphotoreceptor matrix (IPM), with its associated proteoglycans (pink), across the outer limiting membrane (OLM; red spots), and into the outer nuclear layer (ONL; blue cells). Müller glia (yellow) and astrocytes (orange) are quiescent. In the degenerating retinal environment (**B**), Müller glia cells (dark orange) and astrocytes (purple) become activated as a form of neuroprotective response. This activation leads to changes in their morphology, hypertrophy of their processes, and secretion of extracellular molecules, such as CSPGs (pink). These changes appear to be the major factors in determining photoreceptor transplantation efficacy.

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who similarly demonstrated integration, segment formation, and apparent synaptic connectivity with the recipient inner retinal neurons, into the *PDEβ*$^{rd1/rd1}$ (Eberle et al., 2012; Singh et al., 2013), although the resulting morphology of the donor cells is poor (Barber et al., 2013; Singh et al., 2013). Thus disease type, but not severity, is central to determining the transplantation outcome. Although these findings are based on animal models and may differ from clinical presentations, it demonstrates that the recipient retinal environment is an important determinant of how effective transplantation may be. Moreover, it indicates that certain disease types may be more amenable to transplantation than others.

At this point, it is perhaps worth noting an issue of terminology that can complicate the interpretation of many transplantation studies. The outcome of cell transplantation is often described as "integration"; that the donor cells become integrated within the recipient neural retina. This has variously meant that the donor cell bodies are fully incorporated within the neural retina (MacLaren et al., 2006; Pearson et al., 2012) but also situations, particularly at late stages of rapid retinal degeneration, where the recipient ONL has actually disappeared. Here, donor cells do not migrate into the retinal tissue, but remain in the subretinal space (Eberle et al., 2012; Singh et al., 2013), where they might form synaptic contacts to endogenous second-order neurons. It is important that such distinctions are clearly made when reporting transplantation outcome and readers bear such distinctions in mind when comparing reports.

Retinal gliosis and its impact on photoreceptor transplantation

Photoreceptor loss involves a number of concomitant changes in the neural retina, one of the most striking being the process of reactive gliosis by Müller cells. Gliosis is well known to be a limiting factor in the regeneration of other areas of the CNS such as the spinal cord. Like elsewhere in the CNS, the glial scar in the retina may represent a physical barrier to cell migration or act as a reservoir of inhibitory ECM molecules or a combination of these (Ridet et al., 1997). In the CNS, gliosis comprises a complex sequence of events including macrophage infiltration and the proliferation of oligodendrocytes, microglia, and astrocytes. Astrocytes become hypertrophic, upregulate the intermediate filament proteins such as glial fibrillary acidic protein (GFAP) and vimentin, and secrete various ECM components. The retina lacks oligodendrocytes; however, astrocytes are located in the inner surface at the ganglion cell layer (GCL), and Müller glial cells reside in the inner retina, with processes spanning from the inner retina to the OLM. During retinal degeneration or injury, including retinal detachments, both types of glial cells undergo reactive gliosis. The upregulation of GFAP and vimentin typically originates in the end foot region of the Müller cell at the vitreous side of the retina, extending up into the apical processes forming lateral branches. In addition, increasing GFAP and vimentin, reactive Müller cells may also undergo hypertrophy, presenting a proliferation of processes at the outer edge of the retina, which can extend beyond the limits of the OLM (Anderson et al., 1986; Lewis & Fisher, 2000, 2003). Studies in the brain suggest that intermediate filaments play a structural role to allow the process of glial hypertrophy and stabilization of the fragile tissue after injury (Eng & Ghirnikar, 1994; Jones & Redpath, 1998). In advanced glial scar formation, where extensive photoreceptor cell death ensues, a fibrous seal forms between the retina and RPE to allow remodeling of the inner retinal neurons (Jones & Marc, 2005).

Glial scarring has been proposed to explain the lack of integration of retinal sheets with the host retina, as neurite extension does not occur in regions of reactive gliosis (Zhang et al., 2003). It is also highly likely to present a similar obstacle to the migration and integration of dissociated cells transplanted into the subretinal space. For example, hippocampus-derived neuronal progenitors integrated poorly in the adult retina of dystrophic rats compared with non-dystrophic animals (Young et al., 2000). We have similarly observed an inverse correlation between the level of GFAP/vimentin staining in mouse models of degeneration and the level of cell integration following photoreceptor precursor cell transplantation (Barber et al., 2013). Notably, two of the models examined by Pearson and colleagues, *Prph2*$^{+/\Delta 307}$ and *PDE6β*$^{rd1/rd1}$, actually presented a withdrawal of GFAP^{+ve} Muller glial fibers from the ONL to the OPL as degeneration progressed (Barber et al., 2013; Pearson, Hippert and Graca, unpublished observations). These models also permitted improved integration of transplanted donor cells at later, compared with earlier, stages of degeneration (Barber et al., 2013; see also Kwan et al., 1999). Support for an inhibitory role played by gliosis is also provided by the study of Kinouchi et al. (2003). They compared the ability of cells transplanted into the subretinal space to migrate into the retinae of wild-type mice with those of mice deficient in GFAP and vimentin (*GFAP*$^{-/-}$*Vim*$^{-/-}$). Mice deficient in both GFAP and vimentin have reduced levels of glial scarring after CNS injury. The group also found that transplanted cell migration into the various retinal layers and donor cell neurite outgrowth were both significantly increased in *GFAP*$^{-/-}$*Vim*$^{-/-}$ mice compared to wild-type recipients, suggesting that reactive Müller glia and astrocytes act as barriers to the movement of cells into the retina. There are some confounding aspects in interpreting these results; however, the *GFAP*$^{-/-}$*Vim*$^{-/-}$ mouse has also been reported to have disrupted limiting membrane and a prevalence for detachment and shearing (Verardo et al., 2008), all of which may contribute to enhancing donor cell access to the recipient retina (see section on OLM). Nonetheless, gliosis has been reported to have a negative impact on viral transduction efficiency; Calame et al. (2011) demonstrated that lentiviral cell transduction was negatively affected by the extent of reactive gliosis in the recipient retinal environment, highlighting the importance of gliosis in other therapeutic strategies.

Confusingly, gliosis has been reported to have beneficial as well as negative effects on transplantation strategies; Nishida et al. (2000) reported that upregulation of intermediate filament proteins following retinal damage may actually promote the survival of transplanted neuronal stem cells. In their study, they showed that in regions with increased levels of GFAP, survival (although not necessarily integration) of transplanted stem cells was better in comparison with normal, undamaged retinas. Lee et al. (2011) found that glial processes of reactive Müller glia form "rings," which contribute to the survival of remaining cone photoreceptors. It has yet to be determined whether gliosis protects the long-term survival of transplanted donor cells, should they manage to successfully integrate within the degenerating recipient retina.

Deposition of ECM components in degeneration and impact on photoreceptor transplantation

The ECM is a complex network of scaffolding molecules that play an important role in cell signaling, migration, and tissue structure. In the CNS, the ECM is important for proper development and organization of the neuronal structures as well as promoting the survival of neurons and axons. Following CNS insult, the composition of the extracellular environment is altered, resulting in the expression of both growth-inhibitory and growth-stimulatory factors.

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Three classes of molecules have been found to impair neuronal regeneration: chemorepulsive guidance molecules, myelin-associated growth inhibitors, and CSPGs (McGee & Strittmatter, 2003; He & Koprivica, 2004).

In the injured CNS, several repulsive axon guidance molecules, such as Semaphorin 3A and 5A, Ephrin-B3, and Ephrin-A4, become upregulated (Kantor et al., 2004; Kaneko et al., 2006; Pasterkamp & Verhaagen, 2006); this re-expression of negative guidance molecules at the site of the injury may be a factor in limiting the capacity of damaged axons to re-elongate their processes. The role of such molecules in retinal photoreceptor transplantation has yet to be explored. CNS regeneration is also often compromised by the presence of myelin and associated inhibitory proteins such as Nogo-A, which prevents the passage of regenerating growth cones (Bandtlow & Schwab, 2000). Since the neural retina lacks myelin, such problems are not faced by the transplanted photoreceptor.

The retinal environment, however, like elsewhere in the CNS, is enriched in proteoglycans (PGs). PGs are major components of the ECM in the brain and consist of a protein core with one or more glycosaminoglycan (GAG) chains attached. GAGs are classified into four families: heparan sulfate (HS), chondroitin sulfate (CS), dermatan sulfate (DS), and keratan sulfate (KS) and PGs are commonly classified according to their associated GAG chains (e.g., CSPGs) but also by the core protein properties. Neurocan, versican, brevican, and aggrecan are CSPGs of the CNS. In the eye, CSPGs predominate in the neural retina, while HSPGs are expressed throughout the basement membranes (Inatani & Tanihara, 2002). CSPGs bind many different ECM proteins and growth factors making them important players in a variety of regulatory processes including cell adhesion, migration, and differentiation. In the eye, both CSPGs and HSPGs are important in determining axonal guidance from the retina (Ichijo, 2004). CSPGs are upregulated in response to injury and disease throughout the CNS (Inatani et al., 2000; Fawcett, 2009; Gumy et al., 2010) and participate in the inhibition of axon regeneration, mainly through their GAG side chains (Friedlander et al., 1994; Gilbert et al., 2005; Crespo et al., 2007). It is thought that CSPGs act as guidance cues in development and injury, working as boundary cues for neuronal remodeling (Friedlander et al., 1994; Fawcett, 2009). However, our understanding of changes in their expression and their role in retinal degeneration is more limited. Clinically, PGs may be implicated in the pathogenesis of AMD, and poor binding of the disease-associated 402H variant of complement factor H to PGs in Bruch's membrane may provide a potential disease mechanism for AMD (Clark et al., 2010*a*,*b*). With respect to transplantation, they are likely to present a repulsive barrier to transplanted donor cells. Pearson and colleagues have reported very different patterns of CSPG expression in different models of degeneration, as assessed with the broad-spectrum CSPG antibody CS-56 (Barber et al., 2013; Pearson, Hippert and Graca, unpublished observations). Qualitative assessments revealed that the intensity of CS-56 staining was inversely correlated with the number of integrated donor cells post-transplantation (Barber et al., 2013).

The role of specific CSPGs in photoreceptor transplantation is at present unknown. A recent study showed that aggrecan is significantly increased in two rat models of retinal dystrophy, particularly around the IPM (Chen et al., 2012), while microarray analysis of individual Müller glial cells from a mouse model of RP identified a significant increase in CSPG5 (neuroglycan) (Roesch et al., 2012). One of the most studied proteoglycans in the retina is neurocan. Neurocan expression is increased in retinae injured by transient ischemia, a response apparently mediated by Müller glia (Inatani et al., 2000), and in the *PDE6β*$^{rd1/rd1}$ mouse and RCS rat models of retinal degeneration (Inatani et al., 2001). Neurocan is reported to restrict neurite outgrowth in neuronal cell cultures, while retinal axons from embryonic rat retinal explants are repulsed in regions of the brain where neurocan is enriched (Friedlander et al., 1994; Tuttle et al., 1998; Asher et al., 2000). Similarly, Inatani et al., 2001 have shown that the neurite outgrowth of cultured retinal ganglion cells (RGCs) is inhibited on neurocan-coated plates. Moreover, they found that this inhibition persists even after the removal of chondroitin sulfate GAG chains from the core protein, suggesting that the neurocan protein itself is inhibitory. Li et al. (2000) observed that binding of neurocan to its receptor impedes both N-cadherin and β1-integrin mediated adhesion, which inhibits the neurite outgrowth extension from retinal neuronal cells (Li et al., 2000). It is not yet known whether neurocan influences transplanted photoreceptor precursor migration and integration.

Strategies that prevent the formation of inhibitory PGs, or break them down, are of significant interest for improving the outcome of cell regeneration and replacement strategies. Fluorinated glucosamine analogs block the synthesis of chondroitin sulfate sugar chains by preventing elongation of polysaccharide chains. Conversely, the bacterial enzyme chondroitinase ABC (ChABC) degrades GAG chains into disaccharides, leaving the core protein largely intact. ChABC has been reported to promote functional recovery (Bradbury et al., 2002; Crespo et al., 2007) and structural plasticity (Fawcett, 2009) after spinal cord damage. These principles have been applied in the retina with encouraging results; numerous studies with both stem cell and photoreceptor precursors transplants have demonstrated that treatment with ChABC prior to the transplantation increased the number and survival of integrated cells (Suzuki et al., 2007; Ma et al., 2011; Barber et al., 2013).

Currently, local treatment with ChABC is the major strategy to override the inhibitory effect of the CSPGs on the neuronal regeneration. However, there are disadvantages in using it as a therapeutic treatment in patients, including its incomplete removal of GAG chains from all core proteins (Lemons et al., 2003), the likely need for repeated applications and the potential for an inflammatory reaction due to its bacterial origin (see Lee et al., 2010). Therefore, it is important to look for other strategies that would overcome the negative impact of CSPGs on regeneration. Upregulation of the proteases that are involved in the turnover of the proteoglycans could be an alternative method to reduce levels of the CSPGs in the extracellular environment. Matrix metalloproteases (MMPs), which remove both the core protein and the GAG chains, can help to degrade the CSPGs such as NG2 within glial scar and enhance neuronal repair (Larsen et al., 2003; Pizzi & Crowe, 2007). The role of MMPs in photoreceptor transplantation is discussed below.

Matrix metalloproteases

The MMPs are a family of proteolytic enzymes that degrade components of the ECM including collagen, laminin, fibronectin, and proteoglycans such as CSPGs. MMPs play important roles in CNS development, including neurogenesis, cell migration, and axon guidance. Two MMPs, gelatinase A (MMP-2) and gelatinase B (MMP-9), have been shown to be particularly important in the central and peripheral nervous systems in the regulation of neurite outgrowth, both in injury and development. Both types are expressed by retinal Müller cells.

A number of studies have demonstrated how cell migration might be improved *via* the manipulation of MMPs. As mentioned previously, adult-derived hippocampal neural stem cells (AHSCs)

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can migrate effectively into a recipient neural retina, while embryonic retinal progenitors do not. By comparing these two populations *in vitro*, Suzuki et al. (2006) demonstrated that a key difference between them is the expression of MMP-2, with expression being markedly higher in the AHSC population. Moreover, the migratory capacity of these cells could be abrogated by treatment with an MMP-2 inhibitor and stimulated *via* exogenous activation of MMP-2. Similarly, EPO has been reported to upregulate the expression of MMP-2 within the CNS (Wang et al., 2006). When simultaneously administered with an immortalized human Muller glial cell line, MIO-M1, it led to a modest increase in the number of Müller cells migrating into the recipient neural retina (Singhal et al., 2008). Finally, a mouse model of autoimmune disease, the MRL/MpJ mouse, has an unusual capacity to undergo scarless regeneration of cardiac and ear tissue. Investigations in the retina of these mice have shown elevated levels of MMP-2 and MMP-9 expression and decreased levels of scar-related inhibitory molecules such as neurocan and CD44. When retinal explants from the MRL/MpJ mouse were cultured with donor retinal progenitor cells, the MRL/MpJ mouse permitted greater levels of cell migration compared with that observed in both wild-type and $PDE\beta^{rd1/rd1}$ mice (Tucker et al., 2008). Further circumstantial evidence for a role of MMPs in promoting migration and plasticity comes from a study by Young and colleagues, who reported better integration of retinal progenitor cells in a model of laser injury, compared with uninjured adult wild-type recipients. In the same study, they found that focal levels of MMP2, MMP9, and CD44 were upregulated, while neurocan was reduced, around the site of laser injury, although no direct causal link was established (Jiang et al., 2010). It will be particularly interesting to determine whether MMPs play a role in the migratory capabilities of photoreceptor precursors.

The findings summarized above suggest that manipulation of MMP-2 levels might trigger and/or enhance the migration and integration of transplanted cell populations into the recipient retina. MMP expression can be regulated at a number of levels including gene transcription, proenzyme activation by membrane-type metalloproteases (MT-MMPs), and inhibition by tissue inhibitors of MMPs (TIMPs). Increasing MMP-2 levels in the recipient retina provide an intriguing avenue of investigation in the development of retinal transplantation therapy. However, such strategies are not without their drawbacks, as MMP activity is detrimental in the early stages of the injury response, promoting inflammation and apoptosis (Parks et al., 2004). Even at later stages, increasing MMP activity is not without its difficulties, since CSPGs play an important role in maintaining the adhesion between photoreceptors and the overlying RPE. Enzymes that digest such molecules may also lead to increased retinal detachment (Hageman et al., 1995). Devising a therapeutic strategy involving the degradation of ECM molecules therefore presents a difficult balancing act.

The outer limiting membrane

In the opossum, transplanted cells are able to migrate and integrate within juvenile but not adult recipient retinae (Sakaguchi et al., 2003, 2005). This change coincides with the maturation of Müller glial cells, which, amongst other functions, form anatomical barriers within the host retina, including the OLM. The OLM comprises a series of zonula adherens junctional complexes between the end feet of the Müller glia and photoreceptor inner segments. It is first discernible around P5 in the mouse (Uga & Smelser, 1973), suggesting it may present a physical barrier to cell integration in the mature retina. Adherens junctions are composed of transmembrane cadherin–catenin complexes that mediate cell–cell adhesion and form a diffusional and physical barrier between the subretinal space and the photoreceptor cell bodies. These complexes interact with a cytoplasmic plaque comprised of adaptor proteins, including ZO-1 and Crb1 (Mehalow et al., 2003), which are essential for adherens junction formation and stabilization. Recessive mutations in the human CRB1 gene cause retinal diseases including RP and Leber congenital amaurosis (Meuleman et al., 2004; van de Pavert et al., 2007). A mouse model of retinal degeneration, $Crb1^{rd8/rd8}$, has a single base deletion in the Crb1 gene, and presents a progressive retinal degeneration together with an increasingly fragmented OLM (Mehalow et al., 2003). Transplanted photoreceptor integration is markedly enhanced in these animals compared with wild-type controls (Pearson et al., 2010). However, in models of retinal degeneration caused by other gene defects, the OLM may undergo remodeling but remains largely intact (Barber et al., 2013; Pearson, Hippert and Graca, unpublished observations; also see Campbell et al., 2006). This suggests that the OLM would remain a significant barrier to transplanted photoreceptor cell integration in the majority of retinal degenerations. Using a pharmacological toxin, alpha-aminoadipic acid (AAA), to disrupt Müller glial function and, indirectly, OLM integrity, we provided evidence to suggest that disrupting the OLM improves cell integration into the adult wild-type retina (West et al., 2008). However, such strategies are unsuitable in degenerate retinae due to the toxic effects on the supportive Müller glia. We therefore examined an alternative method to induce transient OLM disruption using small interfering RNA (siRNA) to promote transcriptional gene silencing of relevant OLM-related proteins. By combining the knockdown of the adherens junction adaptor protein, ZO-1, with cell transplantation, we subsequently demonstrated that it is possible to invoke a reversible disruption in OLM integrity that permits significantly increased levels of photoreceptor precursor cell integration into both the wild-type and degenerating neural retina (Pearson et al., 2010; Barber et al., 2013). While these experiments demonstrate an important proof of concept with regard to the role of the OLM, neither AAA nor ZO-1 RNAi represents ideal therapeutic strategies. As noted above, AAA is a toxin and compromises Müller glial function (Karlsen, 1978), while ZO-1 is expressed not only in the neural retina but also in the RPE and its knockdown may lead to detrimental effects on RPE function, including RPE de-differentiation and proliferation (Sourisseau et al., 2006). Thus, identifying targets whose expression is restricted to the OLM and not the RPE junctional complex could be of significant interest.

In the study by MacLaren et al. (2006), the integration efficiency of the rod-photoreceptor precursors into the immature recipients was similar to that observed following transplantation into adult recipients. One might have anticipated that more cells would integrate within the immature neuroretina as this environment is still enriched in endogenous migratory cues that are used during retinal development and the OLM is not fully developed at this point (Olney, 1968). One explanation for why no difference was observed could be that adult eye is much larger than the P0-P1 eye and is able to accommodate the introduction of 1–2μl of cell suspension more readily. In keeping with this, we have observed a higher degree of suspension reflux following injection into the immature, compared to the adult, eye (unpublished observations). Although speculative at this stage, it is also possible that further cells do integrate into the immature recipient retina, but die as a result of developmental mechanisms.

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Transplanted cell survival

A feature common to all retinal degenerations is photoreceptor cell death and the subsequent activation of the resident macrophage population is called microglia. The presence of increased numbers of macrophages shortly after cell transplantation is associated with significantly fewer integrated photoreceptors in wild-type mice (West et al., 2010). Why they should be present in some but not other transplantations is not clear, but is likely to be linked to the degree of trauma associated with the injection procedure itself. It is not yet known whether macrophages prevent precursor cell integration itself or cause the subsequent destruction of new photoreceptors after they have integrated. Either way, macrophages may present a significant problem for transplantation into the degenerate retina and their subretinal accumulation could be a specific hindrance to cell transplantation in certain diseases.

Activated microglia produce a number of factors that are detrimental to cell survival including IL-1β, IL-6, nitric oxide, TNF-α, and reactive oxygen species. While some of these factors have been implicated in progenitor cell migration, they are most usually associated with cell death and, importantly for strategies involving the transplantation of undifferentiated stem cells as opposed to precursor cells, have also been shown to inhibit neurogenesis (Monje et al., 2003). Macrophages have been observed in both the *rd* and *rds* mouse models of RP and an experimental model of autoimmune uveoretinitis (Jiang et al., 1999; Hughes et al., 2003; Jiang et al., 2006) and in the retinae of *rd1* and *rds* mice following precursor cell transplantation (Sancho-Pelluz et al., 2008). This increased inflammatory status of degenerate retinae may prompt a premature rejection of transplanted cells.

More than 20 years ago, Jiang and Streilein (1991) reported that neonatal neural retina survived in the subretinal space of recipient mice that were of the same genetic background as the donor, but were slowly rejected when placed in non-matched recipients, indicating that immune factors were likely to be responsible for the rejection. Immune rejection is a major problem in many transplantation paradigms and an in-depth discussion is beyond the scope of this review. Briefly, however, the eye is frequently described as an immune-privileged site, a site that allows foreign grafts to survive for extended to indefinite periods of time without rejection. The neonatal neural retina itself has been defined as partially immune-privileged, since it can survive when grafted into non-immune-privileged sites, such as the kidney capsule. This is in contrast to non-immune-privileged tissue types, such as skin, which is rejected shortly after transplantation, and fully immune-privileged tissue, such as the cornea, which survives for indefinite periods of time. Although often regarded as immune privileged, the adult neural retina contains immune-competent cells capable of recruiting powerful immune regulatory networks (Dick, 2012). Of particular importance to photoreceptor precursor cell transplantation is the immunological status of the subretinal space, the preferred site for photoreceptor transplantation. The subretinal space has been described as having partial immune privilege. It displays two important features of immune-privileged sites; it protects allogeneic tissue grafts from immune rejection and it promotes the acquisition of systemic immune deviation to antigens placed in this site, a form of immune tolerance mediated by antigen-specific T regulatory cells. These cells are produced in the spleen and suppress the host immune reactions to alloantigens present in the anterior chamber of the eye (see Wilbanks & Streilein, 1990; Streilein et al., 2002).

The partial immune privilege of the subretinal space has significant implications for photoreceptor transplantation. A number of tissue sources are under consideration for photoreceptor replacement therapy, including ES cells, dissociated cells either from acute tissue isolation or from cultured populations, tissue fragments, and whole sheet transplantation. These all display different immune profiles. Moreover, immune deviation is lost if RPE cell viability is compromised or the outer blood-retinal barrier is disrupted (Wenkel & Streilein, 1998). Such barriers are often compromised in degenerating or injured retinae. Moreover, the transplantation procedure itself introduces an additional disruption, highlighting the importance of keeping surgical trauma to a minimum (see Pearson et al., 2012).

The majority of investigations into transplanted cell survival in the retina have focused on the transplantation of sheets or fragments of retinal tissue. Neonatal retinal allografts survive in the subretinal space for extended periods of time (up to 1 month), but eventually deteriorate. This deterioration appears to coincide with the loss of immune deviation and the onset of donor-specific delayed hypersensitivity (Jiang & Streilein, 1991; Jiang et al., 1993, 1995; Ghosh et al., 2000). Streilein et al. (2002) suggest that this may be due to the microglial population, which are resident in the retinal grafts at the time of transplantation and can act as potent antigen-presenting cells. Similarly, tissue fragments appear to be promptly rejected, regardless of allo or xenogeneic status while full-thickness allogeneic embryonic retinal grafts can survive for prolonged periods in the adult recipient (Ghosh et al., 2008). Adult retinal sheets can also survive in the subretinal space for several months, although older grafts presented a loss of retinal lamination and there is little evidence of synaptic connectivity between graft and recipient (Ghosh et al., 1999, 2000, 2008). Further analysis demonstrated the presence of major histocompatibility complex (MHC) class I and II proteins on transplanted fragmented retinal tissue but not retinal sheets, suggesting that host immune responses to fragmented and intact retinal transplants might be different (Ghosh et al., 2000).

Few studies have examined the survival of retinal cells transplanted to the subretinal space, although a number of studies have examined long term neural stem/progenitor cell transplantation. Interestingly, cultured neural progenitors appear to be less immunogenic, compared with freshly dissociated neural progenitors, even following xenotransplantation (Ma & Streilein, 1998; Hori et al., 2003). Similarly, others have demonstrated good survival of cultured retinal allografts in pigs at 10 weeks post-transplantation (Warfvinge et al., 2006), although long-term follow-ups are required. Neurons do not generally express major histocompatibility complex (MHC) proteins and as such might be expected to avoid recipient sensitization. However, they may express minor histocompatibility antigens, which can be released upon cell death and captured by host antigen-presenting cells. More importantly, the retina also includes significant glial populations, including Müller glia and astrocytes, both of which do express MHC1. It is possible that cultured cells fair better than acute dissociations or fragments because of the absence of these microglia populations.

A major limitation of the studies described above is that very few of the donor cells correctly integrated within the recipient ONL and the survival of transplanted cells was evaluated by examining the mass of cells present in the subretinal space (Wojciechowski et al., 2002; Warfvinge et al., 2006). By transplanting postmitotic photoreceptor precursor cells, we assessed the ability of integrated transplanted photoreceptors to survive within recipient retinas with partially mismatched haplotypes. We found that transplanted photoreceptor cells integrated within the adult mouse retina are subject to a delayed host immune response from around 4 months

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post-transplantation. However, they can survive for extended periods of time, up to a year, provided these immune responses are modulated (West et al., 2010). In a minority of eyes, we observed a marked reduction in integrated photoreceptor number at 1 month post-transplantation. These were always correlated with significant numbers of ameboid macrophages, indicating an acute inflammatory response. More recently, we have observed high levels of inflammatory markers in the 72 h immediately following transplantation in the majority of transplants, but these resolve in the majority of cases by 3 weeks post-transplantation (Warre-Cornish et al., 2013; Pearson, unpublished observations). In order to examine immune responses in relative isolation, these experiments were performed in the wild-type mouse. However, as noted above, retinal degeneration often associated with the activation of resident microglia (Roque et al., 1996; Hughes et al., 2003). The difference in inflammatory status between normal and degenerate retinas may thus have a significant impact on integrated photoreceptor cell survival and is an area that warrants further investigation.

The eventual rejection of transplanted tissue reported thus far raises obvious concerns for the durability of photoreceptor replacement strategies. As noted above, the most likely source of donor cells are postmitotic precursor (i.e., partially differentiated) cells derived from either embryonic or induced pluripotential stem cells. Immunological maturity occurs relatively late in development, after the point at which ES cells are harvested. However, these cells can express low levels of MHC protein class 1, which may be sufficient to induce chronic rejection (Drukker et al., 2002). Moreover, the majority of transplantation strategies involves the partial differentiation of the ES cells prior to transplantation, during which they are likely to begin to express MHCs. One strategy to protect hESC-derived cells from immune rejection is the use of immunosuppressive drugs. However, apart from their general toxicity, these drugs also cause general immunosuppression and can therefore increase the risk of infection and cancer. It may be possible to induce immune tolerance of hESC-derived cells by disrupting the co-stimulatory pathways required for the activation of T lymphocytes (Felix et al., 2010), the major immune cell type responsible for rejection. In support of this notion, an impressive recent study has shown that it is able to create a local immunosuppressive milieu around the transplanted hESC-derived by inhibiting the activation of the T-cells. This apparently protects the hESC-derived cells from allogenic human immune responses *in vivo* (Rong et al., 2014). A key advantage of iPSCs for human cell therapy is that patient-specific iPSCs are autologous, and, therefore, it has been assumed that the cells derived from them can be transplanted into the same patient without concerns over immune rejection. However, in contrast to this assumption, a recent study using cells differentiated from iPSCs within teratomas were immunogenic when transplanted into syngeneic C57Bl/6 recipient mice, raising concerns about the immunogenicity of iPSCs (Zhao et al., 2011). Until more is known about the immunogenicity of iPS and ES cells, it would therefore seem prudent to continue to explore the therapeutic potential of both as potential donor cell sources for photoreceptor replacement.

Improving integration by optimizing the photoreceptor transplantation procedure

Methodological factors affecting transplantation outcome include cell reflux, a persistent problem particularly when delivering a bolus injection of cells into an enclosed pressurized space such as the eye; clumping and death of transplanted cells; and degeneration of the recipient photoreceptor layer caused by prolonged physical separation from the RPE by the injected cell mass. The majority of experimental protocols uses one of the two methods of cell transplantation: subretinal and intravitreal. The subretinal injection is more technically demanding and involves a more significant disruption to the retina architecture. However, there is a natural anatomical cleavage plane between the photoreceptor layer and the RPE, into which cells can be placed with little trauma. Such strategies have been used successfully for the administration of viral vectors for gene replacement therapy in patients (Bainbridge et al., 2008; Maguire et al., 2008). Subsequent re-attachment of the retina after relatively short periods usually results in a rapid return to normal vision. Work in the porcine large animal model has confirmed that xenograft retinal precursor cells can be transplanted directly into the retina *via* the subretinal space with minimal trauma (Warfvinge et al., 2006). Intravitreal injection is considered to be less invasive and easier to perform, however the transplanted cells must migrate across the densely packed ganglion cell fiber layer before crossing the whole neural retina to reach the photoreceptor layer. Cells with highly migratory properties, such as ES cells or those derived from neural stem cells have been reported to enter the retina after intravitreal injection, but their ability to specifically target the photoreceptor layer is limited (Meyer et al., 2006).

Perhaps unsurprisingly, the number of donor cells transplanted per injection has a significant impact on the number of cells integrating, but importantly there is a trade off with regard to the survival and integrity of the recipient retina. If too many cells are transplanted, they may remain as a large mass in the subretinal space, separating the underlying neural retina from the RPE, which can lead to further degeneration (Pearson et al., 2012). Klassen et al. (2008) found that cell reflux in the larger animal model can be prevented to some extent by the addition of an air bubble into the subretinal injection suspension, although we have also found the presence of air in subretinal detachments to lead to an exacerbated immune response (Pearson, unpublished observations). Similar surgical manipulations, including scleral puncture and multiple injections improve both the number of successful transplantations, and the number of integrated cells (Pearson et al., 2012). In our experience in the mouse, by far the greatest improvement was observed following fluorescent-activated cell sorting (FACS) of the donor cell population, to ensure that a purified population of photoreceptor precursor cells is transplanted (Pearson et al., 2012). In Pearson et al. (2012), FACS-sorted rod-photoreceptor precursors were taken from postnatal day (P) 4–8 Nrl.GFP donor mice and injected subretinally to both the superior and the inferior retina. This resulted in a 20- to 30-fold increase in the number of integrated rod photoreceptors compared with previous studies. A multiple injection approach is feasible in the clinical scenario, as has been demonstrated in one of the RPE65-related LCA retinal gene therapy clinical trials (Jacobson et al., 2012). To date, the two-site injection protocol has proved to be safe in both adults and children. While examining strategies to improve transplantation efficacy, Pearson, Ali and colleagues also observed that a single subretinal detachment prior to the photoreceptor transplant leads to a 2.4-fold increase in integration efficiency (Pearson et al., 2010, 2012). Although untested, this is likely to be related to other studies that have demonstrated a beneficial role of acute injury in transplantation outcome (Nishida et al., 2000; Kurimoto et al., 2001). It is well known that subretinal detachment stimulates the release of growth factors and cytokinesis (Nakazawa et al., 2006), some of which may promote survival and migration of the donor cells within the recipient retina. Interestingly, Fisher & Lewis (2003) reported that

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neuronal reattachment following a subretinal detachment is known to induce outer segment regrowth and outgrowth of rod axons into the inner retina. However, attempts to combine the predetachment procedure with multiple injections at the time of transplantation led to an unacceptably high level of transplant failure and recipient retinal injury (see Pearson et al., 2012, supplementary information).

Conclusions

In summary, the prospects for photoreceptor replacement therapy look very promising. The last decade has seen major advances including the demonstration of restoration of visual function following photoreceptor transplantation and the generation of transplantable donor cells from renewable sources. We have also seen that it is possible to transplant cells into even very severely diseased retinae. Nonetheless, many challenges remain. The efficiency of donor cell integration after transplantation into the diseased retina must still be improved. Not all degenerations are the same and each presents its own set of barriers that may impede the passage of donor cells from the site of transplantation into the recipient retina. Once there, these cells may face challenges from the host immune system. Moreover, much of the work reported to date has focused on rod transplantation in animal models, typically the mouse. While it makes every sense to continue with this experimental model in order to identify the limitations of photoreceptor transplantation and to identify strategies to overcome them, it is very important that we also move forward. Major topics of interest include the potential to restore photopic vision by the transplantation of cone photoreceptors, those most important for human vision, and the establishment of protocols for the generation of photoreceptors from human ES and iPS cell sources and their robust transplantation and integration. There will undoubtedly be new challenges to address and overcome but the lessons we learn from rod transplantation will be very valuable.

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