Photoreceptor precursors derived from three-dimensional embryonic stem cell cultures integrate and mature within adult degenerate retina

Anai Gonzalez-Cordero1,5, Emma L West1,5, Rachael A Pearson1, Yanai Duran1, Livia S Carvalho1, Colin J Chu1, Arifa Naeem1, Samuel J I Blackford1, Anastasios Georgiadis1, Jorn Lakowski2, Mike Hubank3, Alexander J Smith1, James W B Bainbridge1, Jane C Sowden2 & Robin R Ali1,4

Irreversible blindness caused by loss of photoreceptors may be amenable to cell therapy. We previously demonstrated retinal repair1 and restoration of vision through transplantation of photoreceptor precursors obtained from postnatal retinas into visually impaired adult mice2,3. Considerable progress has been made in differentiating embryonic stem cells (ESCs) in vitro toward photoreceptor lineages4–6. However, the capability of ESC-derived photoreceptors to integrate after transplantation has not been demonstrated unequivocally. Here, to isolate photoreceptor precursors fit for transplantation, we adapted a recently reported three-dimensional (3D) differentiation protocol that generates neuroretina from mouse ESCs6. We show that rod precursors derived by this protocol and selected via a GFP reporter under the control of a Rhodopsin promoter integrate within degenerate retinas of adult mice and mature into outer segment–bearing photoreceptors. Notably, ESC-derived precursors at a developmental stage similar to postnatal days 4–8 integrate more efficiently compared with cells at other stages. This study shows conclusively that ESCs can provide a source of photoreceptors for retinal cell transplantation.

Many studies by our group and others have demonstrated integration into wild-type and degenerate mouse retinas of photoreceptor precursors isolated from early postnatal retinas1,3,7–12. Moreover, we have shown that transplantation of a purified population of post-natal photoreceptor precursors can restore rod-mediated vision in mice9. Both the number of cells transplanted and the stage of their development at the time of transplantation are important parameters in achieving efficient integration1,2. The requisite next step toward clinical translation is to prove that pluripotent stem cell lines, which can provide equivalent transplantation-competent photoreceptor precursors. Although progress has been made in developing protocols for in vitro differentiation of ESCs and induced pluripotent stem cells (iPSCs) toward photoreceptor lineages4–6,13–16, no study has proved that ESCs can give rise to mature photoreceptors bearing an outer segment. A feature of mature photoreceptors, outer segments are formed of stacked membranous discs packed with the visual pigment and enzymes required for phototransduction. They are essential for mediating efficient light-evoked responses. Using an optimized adherent, two-dimensional (2D) culture system that generates retinal cells5,17, we were unable to demonstrate the integration of GFP-labeled mouse ESC–derived photoreceptors after transplantation18. These findings led us to conclude that although current 2D ESC culture systems produce cells expressing a selection of photoreceptor markers, they do not faithfully re-enact developmental processes and are therefore unlikely to provide a robust source of photoreceptor precursors equivalent to those from the developing retina.

In 2011, groundbreaking work6 described a 3D embryoid body–based differentiation protocol that mimicked normal development of embryonic retinal tissue and raised the possibility of generating authentically specified and correctly staged photoreceptors for transplantation6,19. Here we have optimized and scaled up the generation of mouse ESC-derived photoreceptors in 3D synthetic retinal tissue, enabling us to transplant purified populations of photoreceptors from defined stages of development and to investigate the potential of the cells to integrate within the adult recipient retina and to mature into new photoreceptors.

A schematic of in vitro retinal differentiation is shown in Figure 1a. Continuous neuroepithelium–like structures were detected as early as day 5 of differentiation (Fig. 1b). At day 7, the presumptive eye fields evaginated from the embryoid bodies, forming hemispherical optic vesicle–like structures (Fig. 1c). At around day 9, the optic vesicles invaginated to form optic cup–like structures (Fig. 1d,e). Pigmented retinal pigment epithelium cells were first detected at days 11–12 (Fig. 1f). Transparent neuroepithelial structures were still present within the embryoid bodies at later time points of culture (Fig. 1g). Early eye development occurs through a series of morphogenetic events. A region of the diencephalon committed to form the eye,

Received 13 March; accepted 22 June; published online 21 July 2013; doi:10.1038/nbt.2643
known as the eye field, evaginates bilaterally to form optic vesicles. These vesicles then invaginate to form bi-layered optic cups, generating the presumptive retinal pigment epithelium (RPE) and neural retina.

To investigate this progressive retinal differentiation, we quantified the number of embryoid bodies containing eye-field stage, optic vesicle and optic cup-like structures at days 5, 7 and 9 of differentiation (Fig. 1h–j, respectively). Neuroepithelium and optic vesicle–like structures decreased from days 5 to 9 in culture (Fig. 1h,i, P < 0.01 and P < 0.001, ANOVA N = 4, respectively), but the proportion of optic cup–like structures, characterized by a hinge region produced by the inward folding of the neuroepithelium, increased substantially over the same period (Fig. 1j, P < 0.001, ANOVA N = 4). Although an incomplete invagination was observed in some instances, this did not interfere with further neural retinal specification and photoreceptor differentiation, similar to other studies using human ESCs and iPSCs. Unlike a previously developed protocol, we kept embryoid bodies as intact structures, referred to here as whole embryoid bodies (wEBs), for the entire period of differentiation, as manual excision of optic cup–like structures from the embryoid bodies did not allow the scaling-up required to produce large numbers of transplantable photoreceptors. In addition, in contrast to the earlier protocol, wEB cultures were grown under atmospheric oxygen levels (20% O2; 5% CO2). At days 9–12 of differentiation, optic cup–like structures demonstrated apical-basal polarity, with the apical side facing the interior of the wEB. Large numbers of dividing cells were observed, and mitosis occurred at the apical surface (Supplementary Fig. 1).

Similar to eye development in vivo, the majority of cells within the ESC-derived neuroepithelium analyzed at days 7–12 expressed Rax, Pax6 and Vsx2 (Chx10), indicating that they were proliferating retinal progenitor cells (Supplementary Fig. 2a–d). Also similar to development in vivo, co-localization of Pax6 and Mitf was widespread throughout the neuroepithelium of day 7 optic vesicles (Supplementary Fig. 2e–h). Mitf+ retinal pigment epithelium progenitors became progressively restricted to defined proximal portions of the invaginating neuroepithelium by day 12 (Supplementary Fig. 2i,j). Retinal differentiation was further confirmed by RT-PCR analysis of eye-field transcription factors and retinal progenitor cell markers between day 0 and day 16 of differentiation (Supplementary Fig. 2l). From day 14 onward, wEBs were cultured in serum-free conditions and in the presence of retinoic acid and taurine, factors reported to promote rod photoreceptor fate. These conditions increased expression of rod-specific genes compared with both pulse application of retinoic acid and taurine on days 14–16 of differentiation and culturing with fetal bovine serum throughout the culture period (Supplementary Fig. 3).

We next determined whether the retinal progenitor cells generated using our 3D wEB differentiation system were capable of further differentiation into mature retinal cell types, despite the presence of other nonretinal neuronal and glial cell types (Supplementary Fig. 4). At day 26 of culture, markers for ganglion, amacrine, horizontal and bipolar cells were found in a single layer at the basal side of the
optic cup–like structures. Photoreceptors were observed in a well-developed layer resembling the outer nuclear layer (ONL) (Supplementary Fig. 5) and robust expression of a variety of photoreceptor-specific markers was detected (Fig. 1k). By day 24 of culture, increasing from day 20, the majority of cells in the wEB expressed Crx, a marker of postmitotic photoreceptor precursors (Fig. 11–o). All wEBs examined contained at least one neural retina–like region, and these, without exception, expressed markers of photoreceptor differentiation (n > 500 wEBs). Rod photoreceptors were abundant, and very few cones were detected.

We sought to determine how closely ESC-derived photoreceptor development within the 3D system compared with normal photoreceptor development in vivo by analyzing the time-course of expression of a number of photoreceptor-specific proteins in ESC-derived photoreceptors and photoreceptors from wild-type C57Bl/6j postnatal retinas. There is a peak of Crx expression from postnatal day (P) 3 to P6 in the early postnatal retina, which diminishes in more mature photoreceptors24–26. Similarly, in our wEB cultures the number of Crx+ photoreceptor precursors increased markedly between days 20 and 24 and decreased after day 26 (Fig. 2a). The reduction in Crx protein levels was accompanied by a substantial increase in the presence of Rhodopsin and Recoverin (Fig. 2a), placing cells at day 26 of culture at a stage similar to the P4–P6 stage of development (Supplementary Fig. 6). In vivo, both rod α-Transducin (Gnat1) and Peripherin-2 protein levels increased substantially between P8 and P12, coincident with the onset of outer-segment formation (Fig. 2b,c). A similar pattern was observed in vitro; at day 28 there were few positively labeled cells, but by day 36 the majority of cells were rod α-Transducin and Peripherin-2 positive (Fig. 2d,e, respectively).

To further analyze the degree of similarity between the differentiation states of rod photoreceptors derived from ESCs and from postnatal retinas, we compared their gene expression profiles by microarray analysis. We used an adeno-associated viral vector (pseudotype 2/9) carrying a GFP reporter under the control of a Rhodopsin promoter (AAV2/9.Rhop.GFP) (Fig. 2f) to select the rod photoreceptors. AAV2/9.Rhop.GFP-positive (Rhop.GFP+) rods were sorted by fluorescence-activated cell sorting (FACS) at day 26 of culture, day 34 of culture and P12 (Supplementary Fig. 7). The ESC-derived populations (days 26 and 34) expressed genes enriched in transplantation-competent P4 rod photoreceptor precursors24, consistent with photoreceptor cell differentiation. Day 34 cultures showed higher expression of genes encoding structural components of outer segments and phototransduction, such as Gnat1, Rho, Pde6a and Prph2. Hierarchical cluster analyses demonstrated that the day 34 Rhop.GFP+ cells were more mature as they more closely resembled P12 photoreceptors than the earlier-stage day 26 cells (Fig. 2g). Therefore, we sought to establish whether these late postnatal Rhop.GFP+ rods formed outer segments. Peripherin-2, a marker for outer segments in vivo, was found in the segment region of the ESC-derived rods (Fig. 2h,i). However, although ultrastructural examination of day 36 wEBs demonstrated the presence of inner-segment and cilium–like structures, no outer segments were observed (Fig. 2j). Cross-sections of these structures showed inner segments packed full of mitochondria (Fig. 2j,k) and a typical photoreceptor cilium, which contained the 9+0 microtubular arrangement (Fig. 2l). Together, these findings confirm the survival and differentiation of photoreceptors derived by the wEB 3D protocol to a stage equivalent to late postnatal development.

We next examined the capability of wEB ESC–derived photoreceptor precursors to integrate into adult retina and form new mature photoreceptors. To ensure a robust evaluation of integration and maturation, we assessed expression of outer-segment proteins after transplantation in recipient retinas deficient in these proteins. We transplanted ~200,000 Rhop.GFP+ FACS-sorted precursors (days 26–29) by means of subretinal injection into the adult Gnat1−/− mouse, a model of stationary night blindness, which lacks rod function because of the absence of rod α-Transducin phototransduction protein27. Three weeks after transplantation, Rhop.GFP+–sorted photoreceptor precursors had migrated and integrated into the recipient ONL (Fig. 3a). Integrated ESC-derived rods were correctly oriented within the ONL and were usually found in small clusters, a characteristic frequently seen in transplants using donor-derived photoreceptor precursors28–30. Moreover, integrated rods displayed morphological features typical of mature photoreceptors, including inner and outer segments projected toward the host retinal pigment epithelium (Fig. 3a) and rod spherules in the outer plexiform layer (Fig. 3b). The identity and number of the integrated ESC-derived photoreceptors was established by counting GFP+ cells that also expressed rod α-Transducin (Rhop.GFP+/Gnat1+). Integrated rods were found predominantly around the cell mass near the injection site (Fig. 3c). These cells were still present 6 weeks after transplantation.

Transplanted human ESC-derived cultures labeled with GFP viruses have been reported to integrate within adult mouse retinas27,28. The integrated cells resembled mouse photoreceptor cells in size, and outer segments did not form after transplantation in the Crx− /− mouse model26. As we recently demonstrated that it is possible for contaminating viral particles to be injected with transplanted cells and to label endogenous photoreceptors28,30, potentially leading to false-positive results, we formally excluded this possibility in our experiments. To determine the number of virus-labeled host photoreceptors after transplantation, we used a control ESC-derived CBA.YFP+ FACS-sorted neuronal population transduced with an AAV2/9.Rhop.RFP virus and quantified the YFP−/RFP+ photoreceptors. Transplantation of CBA.YFP+/Rhop.RFP− FACS-sorted cells resulted in a negligible number of virally transduced host photoreceptors (8 ± 2 photoreceptors per retina, n = 16). Without FACS we noted significantly greater numbers of viral-labeled host photoreceptors after transplantation (113 ± 29 versus 8 ± 2, photoreceptors; Mann-Whitney U, P < 0.0001, n = 8) (Supplementary Fig. 8a–c). Moreover, we also examined the transplantation of unsorted ESC-derived mixed populations containing Rhop.GFP+ rods. We observed 113 ± 25 GFP-labeled photoreceptors in the recipient ONL. However, only a small percentage of these cells (1 ± 0.6%) were verified to be ESC-derived Rhop.GFP+/Gnat1+ integrated photoreceptors, suggesting that the majority of Rhop.GFP+/Gnat1− cells were endogenous virus-labeled photoreceptors (Supplementary Fig. 8d–e). In contrast, transplantation of a pure FACS-sorted Rhop.GFP+ population showed that ~80% of ESC-derived Rhop.GFP+ integrated rods were also Gnat1+, similar to P4–8 Nrlp.GFP+ donor-derivative transplants (Supplementary Fig. 9). These experiments highlight the importance of stringent controls to identify true ESC-derived integrated photoreceptors.

Early postnatal rod precursors integrate into the host ONL with greater efficiency than do embryonic, late postnatal or adult mature photoreceptors31. To determine whether ESC-derived photoreceptor precursors behave in a similar manner, we transplanted FACS-sorted Rhop.GFP+ cells at stages equivalent to early postnatal (days 26 and 29 in culture) and late postnatal (day 34 in culture) retina. The number of integrated Rhop.GFP+/Gnat1+ photoreceptors from day 26 (420 ± 98 photoreceptors, n = 16) and day 29 (236 ± 44 photoreceptors, n = 19) cultures was significantly greater than that obtained from day 34 cultures (P < 0.05, ANOVA; 30 ± 6 photoreceptors, n = 24).
Figure 2 Time course of photoreceptor genesis in wEB 3D differentiation system. (a) Temporal expression of Crx and Rhodopsin (red and green, respectively) and Recoverin- and Rhodopsin-positive photoreceptors (red and green, respectively) at different time points of culture. 

(b–e) Immunohistochemical analysis for rod α-Transducin (b, d, red) and Peripherin-2 (c, e, red) in P8 and P12 retinas and ESC-derived photoreceptors at day 28 and 36 of culture, respectively. (f) Schematic of viral labeling and FACS. Light image of day 29 wEBs showing areas of neuroepithelium (black arrows). Fluorescent image of Rhop.GFP + wEBs (white arrows). Sections confirmed Rhop.GFP + in the neuroepithelium. Representative FACS plot of Rhop.GFP + photoreceptors (green) selected by flow cytometry. (g) Hierarchical clustering and heat map of 12 photoreceptor-associated transcripts at days 26 and 34 ESC-derived and P12 donor-derived Rhop.GFP + cells. (h, i) Day 36 viral-labeled Rhop.GFP + photoreceptors showing Peripherin-2 (red) at the base of the inner segments. High magnification of a single Rhop.GFP + photoreceptor stained for Peripherin-2 (i, arrowhead). (j) Ultrastructural sagittal section of a day 36 photoreceptor showing inner segment–like structures (black line), cilium-like (dashed line) structures and the lack of outer segment (black arrowhead). (k, l) Representative images showing transverse sections through an inner segment containing many mitochondria (k, asterisks) and a photoreceptor cilium with a 9+0 microtubular organization (l). Nuclei were stained with DAPI (blue). Scale bars, 3 μm (i), 25 μm (a–e, f insert, h) and 200 μm (f).
Figure 3 Integration and connectivity of ESC-derived photoreceptor precursors. (a,b) Rhop.GFP+ integrated photoreceptors showing mature morphology with outer segments (OS) stained for rod α-Transducin (a, inset) and spherule formation in the outer plexiform layer (OPL) (b, arrowhead). INL, inner nuclear layer; ONL, outer nuclear layer; IS, inner segment. (c) Rhop.GFP+/Gnat1+ integrated cells close to the cell mass in the subretinal space (SRS). (d) Histogram showing the number of Rhop.GFP+/Gnat1+ ESC-derived integrated rods from transplants of days 26, 29 and 34 of culture. Error bars, mean ± s.e.m.; ANOVA, *P < 0.05, **P < 0.001. 
(e-g) Integration of ESC-derived Rhop.GFP+/Gnat1+ photoreceptors into the Gnat1+/−/−, Prph2−−/−/− (f) and Rho−−/−/− (g) degenerate models as demonstrated by rod α-Transducin, Peripherin-2 and Rhodopsin segment staining, respectively (red). (h) Rhop.GFP+/Gnat1+ integrated rod spherule in close proximity to bipolar cells (PKCα3, red). (i) Rhop.GFP+ rod spherule localized with ribbon synaptic marker Dystrophin (red). (j,i, insets) High magnification single confocal sections of boxed region (j) 3D confocal image of Gnat1+/−/− retinal flatmount showing Rhop.GFP+ integrated rod stained for rod α-Transducin (red) and synaptic marker Ribeye (purple). (k-m) 3D reconstruction of the integrated rod, highlighting morphology and arrangement of the rod spherule and ribbon synapse (white arrows). (n) Intracellular calcium changes in integrated Rhop.GFP+, Gnat1+/−/− host and WT photoreceptors are similarly evoked by the mGlur8 agonist DCPG and blocked by the specific antagonist CPPG. Error bars, mean ± s.e.m. (o-p) Mean traces (o) of integrated Rhop.GFP+ (white circles) and recipient photoreceptors (yellow circles) shown in (p). N = number of eyes; n = number of cells imaged. Nuclei were stained with DAPI (blue). Scale bars, 3 μm (i,l,m), 5 μm (e-g,j), 10 μm (a,b,h) and 25 μm (c,p).

(Fig. 3d and Supplementary Fig. 9b), indicating that the developmental stage of the donor photoreceptor is important in determining its ability to integrate.

We have recently shown, by transplanting Nrlp.GFP+ rods derived from the early postnatal retina into mouse models of degeneration, that different disease environments have distinct and marked impacts on the morphology of transplanted photoreceptors3. To confirm the identity of the ESC-derived integrated rods in other models lacking endogenous, photoreceptor-specific proteins, and to determine whether ESC-derived precursors can integrate into different disease environments, we transplanted day 29 Rhop.GFP+ cells into two additional models of inherited retinal degeneration. Compared with transplantation into Gnat1+/− mice, far fewer ESC-derived rods integrated into the ONL of 2-month-old Peripherin-2 null mutant (Prph2−−/−/−) mice and 3-week-old rhodopsin knockout (Rho−−) mice (Supplementary Fig. 10). Transplanted rods integrated within the Gnat1+/− recipient formed long outer segments (Fig. 3e). In contrast, integrated cells in the Prph2−−/−/− and Rho−−/− models formed shorter segments (Fig. 3f,g, respectively), consistent with our previous findings in transplanting donor-derived precursors3. Notably, ESC-derived photoreceptors expressed the outer-segment proteins missing in the endogenous rods in each of the respective knockout models examined.

To establish whether integrated ESC-derived rod precursors were able to connect with the existing retinal circuitry, we examined transplanted eyes for the presence of synapses. Integrated Rhop.GFP+ photoreceptors extended basal processes that terminated as round, synaptic bouton–like structures, which were in close proximity to the afferent terminals of PKCα3 rod bipolar cells in the outer plexiform layer (Fig. 3h). These synapse-like structures expressed the rod ribbon synapse markers Dystrophin and Ribeye (Fig. 3i,j). 3D reconstruction of individual integrated cells in retinal flat mounts demonstrated the correct morphology and anatomical localization of integrated Rhop.GFP+/Gnat1+ cells and highlighted the correct spatial alignment and morphology of the ribbon synapse in relation to the rod spherule (Fig. 3j–m and Supplementary Movie 1).
Finally, we assessed whether the transplanted cells could respond to pharmacological stimuli in a manner similar to that of endogenous rods. In the ONL, the metabotropic glutamate receptor mGlurR8 is expressed on photoreceptor presynaptic terminals, and its activation leads to a characteristic decrease in intracellular calcium in these cells^{29,30} (Fig. 3n–p). (S)-3,4-dicarboxyphenylglycine (DCPG), an agonist with high specificity for the mGlurR8 subtype, consistently evoked appropriate decreases in intracellular calcium in both endogenous Gnat1<sup>−/−</sup> rods and integrated ESC-derived Rhop.GFP<sup>+</sup> precursor cells that were virtually indistinguishable from those seen in wild-type rods (Fig. 3n,o), both in their profile and in the proportion of cells responding. In all cases, these decreases could be blocked by the mGlurR8-specific antagonist (RS)-alpha-cyclopropyl-4-phenylphosphonofenylglycine (CPPG) (Fig. 3n,o). Conversely, specific agonists of another glutamate receptor, the N-methyl-d-aspartate (NMDA) receptor, which is expressed by other retinal neurons but not by photoreceptors, had no effect (Fig. 3n).

In this study, we generated optic cup–like structures from 3D-cultured mouse ESCs and isolated from the derived retinas a population of pure photoreceptor precursors capable of integrating and maturing into new photoreceptors within a recipient retina after transplantation. Notably, we establish that rods obtained from stages in culture similar to P4–8 integrate more efficiently than do mature rods expressing later phototransduction markers. Compared with photoreceptors obtained from our previously described 2D method^{18}, the 3D-differentiated cells expressed significantly greater levels of postnatal rod genes (Supplementary Fig. 11), supporting our hypothesis that developmental stage is crucial for photoreceptor integration^{1,18}. Here, the ESC origin of the integrated photoreceptors was verified by detection of proteins absent in the photoreceptors of the recipient retinas in three different disease models. We also confirmed that transplantation of a heterogeneous population of ESC-derived cells leads to greatly reduced numbers of integrated photoreceptors, consistent with our earlier observations in transplanting donor-derived cells^{2,18}.

In our experiments, we transplanted ~200,000 ESC-derived photoreceptor precursors and observed ~0.3% of these cells integrating into the retina. The number of integrated photoreceptors and their morphology were similar to those in our earlier studies using donor-derived photoreceptor precursors^{19,31}. Assessment of visual function will require further optimization to achieve higher numbers of integrating cells. Based on our earlier work, reliable electoretinographic responses are achieved only with rescue of 150,000 functioning rods. Restoration of visual function was demonstrated in the Gnat1<sup>−/−</sup> model containing an average of 25,000 integrated cells (through testing by means of a water maze and an OptoMotry device), yet electoretinographic responses were not detectable even with this number of new cells^2. We find it difficult to reconcile our findings with previous reports of restoration of mouse electoretinographic responses with as few as 3,000 integrated human ESC-derived Nrl<sup>+</sup> cells that do not form outer segments or with transplants of mixed cell populations derived from mouse iPSC^{28,32}.

In conclusion, the 3D culture system described here provides a robust and consistent method of differentiating ESCs into photoreceptor precursors^8. Our data now demonstrate unequivocally that ESC-derived photoreceptor precursor cells have the capability to integrate and mature to form outer segments and synaptic connections after transplantation into the degenerate adult mouse retina. We present clear evidence to support the utility of ESC-derived cells for photoreceptor replacement therapy. Similar 3D protocols have been developed to generate photoreceptors from human ESCs^{33}; future transplantation studies will seek to establish that similar integration can be achieved using human cells.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. ArrayExpress: E-MEXP-3921 and E-MEXP-3922.

Note: Supplementary information is available in the online version of the paper.

ACKNOWLEDGMENTS

This work was supported by the Medical Research Council UK (mr/J004553/1, G0901550), RP Fighting Blindness (GR566), The Miller’s Trust and Moorfields Eye Charity through a generous private donation. A.G.-C. is a Wellcome Trust PhD student (087256/Z/08/Z). R.A.P. is a Royal Society University Research Fellow. J.C.S. is supported by Great Ormond Street Hospital Children’s Charity. R.R.A. is partly funded by the Department of Health’s National Institute for Health Research Biomedical Research Centre at Moorfields Eye Hospital and Alcon Research Institute. We thank A. Eddaoudi, A. Rose and T. Adejumo for FACS assistance; S. Azam and S. Hara for virus purification; S. Sharma for performing the Affymetrix microarray; and P. Munro for EM assistance. The mouse EK.CCE ESC line^{14} (129/SvEv) was a kind gift of E. Robertson. The following mouse lines were kind gifts: Gnat1<sup>−/−</sup> was provided by J. Lem, Tufts University School of Medicine; Prrph<sup>2</sup> was provided by G. Tezel; Rh<sup>−/−</sup> by P. Humphries, Trinity College Dublin and Nrlp.GFP<sup>−/−</sup> by A. Swaroop, University of Michigan.

AUTHOR CONTRIBUTIONS

A.G.-C. and E.L.W. contributed equally to the concept, design, execution and analysis of all experiments and manuscript writing. R.A.P. performed subretinal transplantation and calcium imaging, and contributed to the concept and design of the experiments, funding and manuscript writing. V.D. performed subretinal transplantsations and histological processing. L.S.C., A.G. and J.L. contributed to experimental execution. C.J.C. performed IMaris reconstruction. A.N. and S.J.I.B. provided technical assistance. M.H. performed microarray data analysis. J.W.B., A.J.S., J.C.S. and R.R.A. contributed to the concept and design of the experiments, funding and to manuscript writing.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. MacLaren, R.E. et al. Retinal repair by transplantation of photoreceptor precursors. Nature 444, 203–207 (2006).
2. Pearson, R.A. et al. Restoration of vision after transplantation of photoreceptors. Nature 485, 99–103 (2012).
3. Barber, A.C. et al. Repair of the degenerate retina by photoreceptor transplantation. Proc. Natl. Acad. Sci. USA 110, 154–159 (2013).
4. Lampi, D.A., Karl, M.O., Ware, C.B. & Reh, T.A. Efficient generation of retinal progenitor cells from human embryonic stem cells. Proc. Natl. Acad. Sci. USA 103, 12769–12774 (2006).
5. Osakada, F. et al. Toward the generation of rod and cone photoreceptors from mouse, monkey and human embryonic stem cells. Nat. Biotechnol. 26, 215–224 (2008).
6. Eiraku, M. et al. Self-organizing optic-cup morphogenesis in three-dimensional culture. Nature 472, 51–56 (2011).
7. Bartsch, U. et al. Retinal cells integrate into the outer nuclear layer and differentiate into mature photoreceptors after subretinal transplantation into adult mice. Exp. Eye Res. 86, 691–700 (2008).
8. Lakowski, J. et al. Cone and rod photoreceptor transplantation in models of the childhood retinopathy Leber congenital amaurosis using flow-sorted Cre-positive donor cells. Hum. Mol. Genet. 19, 4545–4559 (2010).
9. Pearson, R.A. et al. Targeted disruption of outer limiting membrane junctional proteins (Crb1 and ZO-1) increases integration of transplanted photoreceptor precursors into the adult wild-type and degenerating retina. Cell Transplant. 19, 487–503 (2010).
10. Lakowski, J. et al. Effective transplantation of photoreceptor precursor cells selected via cell surface antigen expression. Stem Cells 29, 1391–1404 (2011).
11. Eberle, D., Schubert, S., Postel, K., Gorielt, D. & Ader, M. Increased integration of transplanted CD73-positive photoreceptor precursors into adult mouse retina. Invest. Ophthalmol. Vis. Sci. 52, 6462–6471 (2011).
12. Singh, M.S. et al. Reversal of end-stage retinal degeneration and restoration of visual function by photoreceptor transplantation. Proc. Natl. Acad. Sci. USA 110, 1101–1106 (2013).
13. Meyer, J.S. et al. Modeling early retinal development with human embryonic and induced pluripotent stem cells. Proc. Natl. Acad. Sci. USA 106, 16696–16703 (2009).

14. Hirami, Y. et al. Generation of retinal cells from mouse and human induced pluripotent stem cells. Neurosci. Lett. 458, 126–131 (2009).

15. Meyer, J.S. et al. Optic vesicle-like structures derived from human pluripotent stem cells facilitate a customized approach to retinal disease treatment. Stem Cells 29, 1206–1218 (2011).

16. Phillips, M.J. et al. Blood-derived human iPS cells generate optic vesicle-like structures with the capacity to form retinal laminae and develop synapses. Invest. Ophthalmol. Vis. Sci. 53, 2067–2019 (2012).

17. Osakada, F., Ikeda, H., Sasai, Y. & Takahashi, M. Stepwise differentiation of pluripotent stem cells into retinal cells. Nat. Protoc. 4, 811–824 (2009).

18. West, E.L. Defining the integration capacity of embryonic stem cell-derived photoreceptor precursors. Stem Cells 30, 1424–1435 (2012).

19. Ali, R.R. & Sowden, J.C. Regenerative medicine: DIY eye. Nature 472, 42–43 (2011).

20. Martinez-Morales, J.R. & Wittbrodt, J. Shaping the vertebrate eye. Curr. Opin. Genet. Dev. 19, 511–517 (2009).

21. Hyatt, G.A., Schmitt, E.A., Fadool, J.M. & Dowling, J.E. Retinoic acid alters photoreceptor development in vivo. Proc. Natl. Acad. Sci. USA 93, 13298–13303 (1996).

22. Kelley, M.W., Williams, R.C., Turner, J.K., Creech-Kraft, J.M. & Reh, T.A. Retinoic acid promotes rod photoreceptor differentiation in rat retina in vivo. Neureport 10, 2389–2394 (1999).

23. Lombardini, J.B. Taurine: retinal function. Brain Res. Brain Res. Rev. 16, 151–169 (1991).

24. Chen, S. Crx, a novel Otx-like paired-homeodomain protein, binds to and transactivates photoreceptor cell-specific genes. Neuron 19, 1017–1030 (1997).

25. Furukawa, T., Morrow, E.M. & Cepko, C.L. Crx, a novel otx-like homeobox gene, shows photoreceptor-specific expression and regulates photoreceptor differentiation. Cell 91, 531–541 (1997).

26. Blackshaw, S. et al. Genomic analysis of mouse retinal development. PLoS Biol. 2, e247 (2004).

27. Calvert, P.D. et al. Phototransduction in transgenic mice after targeted deletion of the rod transducin alpha-subunit. Proc. Natl. Acad. Sci. USA 97, 13913–13918 (2000).

28. Lamba, D.A., Gust, J. & Reh, T.A. Transplantation of human embryonic stem cell-derived photoreceptors restores some visual function in Crx-deficient mice. Cell Stem Cell 4, 73–79 (2009).

29. Koulen, P., Kuhn, R., Wässle, H. & Brandstätter, J.H. Modulation of the intracellular calcium concentration in photoreceptor terminals by a presynaptic metabotropic glutamate receptor. Proc. Natl. Acad. Sci. USA 96, 9909–9914 (1999).

30. Koulen, P. & Brandstätter, J.H. Pre- and postsynaptic sites of action of mGluR8b in the mammalian retina. Invest. Ophthalmol. Vis. Sci. 43, 1933–1940 (2002).

31. West, E.L. et al. Pharmacological disruption of the outer limiting membrane leads to increased retinal integration of transplanted photoreceptor precursors. Exp. Eye Res. 86, 601–611 (2008).

32. Tucker, B.A. et al. Transplantation of adult mouse iPS cell-derived photoreceptor precursors restores retinal structure and function in degenerative mice. PLoS ONE 6, e18992 (2011).

33. Nakano, T. et al. Self-formation of optic cups and storable stratified neural retina from human ESCs. Cell Stem Cell 10, 771–785 (2012).

34. Evans, M.J. & Kaufman, M.H. Establishment in culture of pluripotential cells from mouse embryos. Nature 292, 154–156 (1981).

35. Gao, G.-P. et al. Rep/Cap gene amplification and high-yield production of AAV in the mammalian retina. Invest. Ophthalmol. Vis. Sci. 43, 18992 (2011).

36. Lamba, D.A., Gust, J. & Reh, T.A. Transplantation of human embryonic stem cell-derived photoreceptors restores some visual function in Crx-deficient mice. Cell Stem Cell 4, 73–79 (2009).

37. Luhmann, U.F.O. et al. Phototransduction in transgenic mice after targeted deletion of the rod transducin alpha-subunit. Proc. Natl. Acad. Sci. USA 97, 13913–13918 (2000).

38. Tschernutter, M. et al. Phototransduction in transgenic mice after targeted deletion of the rod transducin alpha-subunit. Proc. Natl. Acad. Sci. USA 97, 13913–13918 (2000).

39. Lamba, D.A., Gust, J. & Reh, T.A. Transplantation of human embryonic stem cell-derived photoreceptors restores some visual function in Crx-deficient mice. Cell Stem Cell 4, 73–79 (2009).

40. Koulen, P., Kuhn, R., Wässle, H. & Brandstätter, J.H. Modulation of the intracellular calcium concentration in photoreceptor terminals by a presynaptic metabotropic glutamate receptor. Proc. Natl. Acad. Sci. USA 96, 9909–9914 (1999).

41. Koulen, P. & Brandstätter, J.H. Pre- and postsynaptic sites of action of mGluR8b in the mammalian retina. Invest. Ophthalmol. Vis. Sci. 43, 1933–1940 (2002).

42. West, E.L. et al. Pharmacological disruption of the outer limiting membrane leads to increased retinal integration of transplanted photoreceptor precursors. Exp. Eye Res. 86, 601–611 (2008).

43. Tucker, B.A. et al. Transplantation of adult mouse iPS cell-derived photoreceptor precursors restores retinal structure and function in degenerative mice. PLoS ONE 6, e18992 (2011).

44. Nakano, T. et al. Self-formation of optic cups and storable stratified neural retina from human ESCs. Cell Stem Cell 10, 771–785 (2012).

45. Evans, M.J. & Kaufman, M.H. Establishment in culture of pluripotential cells from mouse embryos. Nature 292, 154–156 (1981).

46. Gao, G.-P. et al. Rep/Cap gene amplification and high-yield production of AAV in an A549 cell line expressing Rep/Cap. Mol. Ther. 5, 644–649 (2002).

47. Davidoff, A.M. et al. Purification of recombinant adeno-associated virus type 8 vectors by ion exchange chromatography generates clinical grade vector stock. J. Virol. Methods 121, 209–215 (2004).

48. Luhmann, U.F.O. et al. Differential modulation of retinal degeneration by Ccl2 and Cx3cr1 chemokine signalling. PLoS ONE 7, e35551 (2012).

49. Schernwittner, M. et al. Long-term preservation of retinal function in the RCS rat model of retinitis pigmentosa following lentivirus-mediated gene therapy. Gene Ther. 12, 747–751 (2005).
Production of recombinant AAV2/9 Rhop.GFP/RFP. A pD10/Rhodopsin promoter-GFP or pD10/Rhodopsin promoter-RFP construct containing AAV-2 inverted terminal repeat was used to generate AAV2/9 Rhop.GFP or Rhop.RFP. Recombinant AAV2/9 serotype particles were produced through AAV-2 inverted terminal repeats was used to generate AAV2/9 Rhop.GFP or Rhop.RFP. Recombinant AAV2/9 serotype particles were produced through a previously described tripartite transfection method into HEK293T cells, followed by purification using ion exchange chromatography. Viral particle titers were determined using dot-blot analysis of purified virus DNA and plasmid controls of known concentrations. wEBs were infected at day 22 of culture with 1 × 10^9 viral particles per wEB in retinal maturation medium.

FACS analysis. wEBs were dissociated with 0.25% Trypsin at various time points and FACS-sorted for Rhop.GFP or CBA.YFP/Rhop.RFP cells, and collected in retinal maturation medium containing 10% FBS, for further analysis.

Calcium imaging. Calcium imaging was performed using methods described previously with minor modifications. Briefly, following osmium fixation and ethanol dehydration, the specimens were embedded in araldite and cured at 60 °C. Semithin (0.7 µm thick) and all sections were collected for analysis. Immunohistochemistry. wEBs and eye cups were fixed for 1 h in 4% paraformaldehyde (PFA) and embedded in OCT (Raymond A. Lamb Ltd.). Cryosections were cut (18 µm thick) and all sections were collected for analysis. For immunohistochemistry, sections were blocked in 5% goat serum and 1% bovine serum albumin in PBS. Primary antibody (Supplementary Table 2) was incubated overnight at 4 °C. Sections were incubated with secondary antibody for 2 h at room temperature, washed and counterstained with DAPI (Sigma-Aldrich). Retinal flatmounts were stained by adapting a published protocol. Following dissection, the retinas were placed into 100% methanol overnight, then blocked and stained in 1% BSA (Sigma-Aldrich), 3% Triton X-100 and 5% goat serum. Alexa-Fluor 488, 546 and 633 secondary antibodies (Invitrogen-Molecular Probes) were used at a 1:500 dilution.

Electron microscopy. wEBs were fixed in 3% glutaraldehyde/1% PFA at 4 °C for 48 h and processed, as previously described with minor modifications. Briefly, following osmium fixation and ethanol dehydration, the specimens were embedded in araldite and cured at 60 °C. Semithin (0.7 µm thick) and ultrathin (0.05 µm) sections were cut using a Leica ultracut S microtome fitted with an appropriate diamond knife (Diatome histoknife Jumbo/Ultrathin). Ultrathin sections were collected on copper grids (100 mesh, Agar Scientific), contrast-stained with 1% uranyl acetate and lead citrate and analyzed using a JEOL 1010 transmission electron microscope (80 kV).

Calcium imaging. Calcium imaging was performed using methods described previously with minor modifications. Briefly, whole-mount neural retinas were loaded with Fura-Red AM (15 µM, Molecular Probes)/Pluronic Acid F127 (0.03% w/v, Sigma) in artificial cerebrospinal fluid (ACSF), which contained in mM: 119.0 NaCl, 26.2 HEPES, 11 D-glucose, 2.5 KCl, 1.0 K2HPO4, 2.5 CaCl2, 1.3 MgCl2 for 1 h at 37 °C and then de-esterified in ACSF alone for 30 min at 37 °C. Retinas were transferred to an inverted microscope (SP2, Leica) and held flat under a nylon-strung platinum wire ‘harp’, photoreceptor side down, and perfused with oxygenated ACSF (36 °C) using a pressurized perfusion system (Harvard Apparatus Ltd). Drugs were applied through the perfusion system and included DCPG ((S)-3,4-dicarboxyphénylglycine, 40 µM), CPPG ((R)-α-cyclopropyl-4-phosphonophénylglycine, 100 µM) and NMDA (N-methyl-d-aspartate, 200 µM) (all Tocris). GFP+ cells were located using epiﬂuorescence before taking confocal XY images of DAPI, GFP and Fura-Red AM to conﬁrm the location of GFP+ cells within the recipient ONL. Only cells with a highly condensed nucleus (typical of rods) located within the recipient ONL were included in the analysis. Fura-Red ﬂuorescence was acquired at 4 s intervals and analyzed off-line. NB: when Fura-Red is excited at 488 nm, its emission undergoes an increase in fluorescence.
as \([\text{Ca}^{2+}]_{i}\) decreases. Endogenous GFP− rods were randomly selected from the Hoechst image. Changes in fluorescence were normalized against the fluorescence at time 0 s and a change of >10% above baseline was considered a response. Analysis was performed masked such that only the timing, not the identity of the drug applied, was known at the time of analysis.

**Cell counts.** Counts of integrated cells were taken 3 weeks after transplantation using a fluorescence microscope (ObserverZ.1, Zeiss). The average number of integrated cells per eye was determined by counting all the integrated Rhop.GFP+ cells or rod α-Transducin+ cells in alternate serial sections through each eye. This was doubled to give an estimate of the mean number of integrated cells per eye. Cells were considered to be integrated if the whole cell body was correctly located within the outer nuclear layer, and at least one of the following was visible: spherule synapse, inner/outer processes, inner/outer segments. Animals were omitted from quantification analysis only if there was clear evidence of an injection occurring intravitreally or if no cell mass was evident in the subretinal space. Counts were done masked such that the identity of the transplanted cells was not known.

**Statistical analysis.** All means are presented ± s.e.m., unless otherwise stated; \(N\), number of animals or independent experiments performed; \(n\), number of eyes, embryoid bodies (prior to day 9 of culture) or wEBs (after day 9 of culture) examined, where appropriate. For assessment of integration efficiency, statistical analysis is based on at least three independent transplantation sessions (cell preparation, FACS and transplantation). Statistical significance was assessed using GraphPad Prism 5 software and denoted as \(*P < 0.05\), \(**P < 0.01\) and \(***P < 0.001\). Appropriate statistical tests were applied including \(t\)-test, Mann-Whitney \(U\) and ANOVA with Tukey’s correction for multiple comparisons.