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Transplantation of Reprogrammed Embryonic Stem Cells Improves Visual Function in a Mouse Model for Retinitis Pigmentosa

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Abstract

Background—To study whether C57BL/6J-*Tyr^{c-2j}*/J (C2J) mouse embryonic stem (ES) cells can differentiate into retinal pigment epithelial (RPE) cells in vitro and then restore retinal function in a model for retinitis pigmentosa: *Rpe65^{rd12}/Rpe65^{rd12}* C57BL6 mice.

Methods—Yellow fluorescent protein (YFP)-labeled C2J ES cells were induced to differentiate into RPE-like structures on PA6 feeders. RPE-specific markers are expressed from differentiated cells in vitro. After differentiation, ES cell-derived RPE-like cells were transplanted into the subretinal space of postnatal day 5 *Rpe65^{rd12}/Rpe65^{rd12}* mice. Live imaging of YFP-labeled C2J ES cells demonstrated survival of the graft. Electroretinograms (ERGs) were performed on transplanted mice to evaluate the functional outcome of transplantation.

Results—RPE-like cells derived from ES cells sequentially express multiple RPE-specific markers. After transplantation, YFP-labeled cells can be tracked with live imaging for as long as 7 months. Although more than half of the mice were complicated with retinal detachments or tumor development, one fourth of the mice showed increased electroretinogram responses in the transplanted eyes. *Rpe65^{rd12}/Rpe65^{rd12}* mice transplanted with RPE-like cells showed significant

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visual recovery during a 7-month period, whereas those injected with saline, PA6 feeders, or undifferentiated ES cells showed no rescue.

Conclusions—ES cells can differentiate, morphologically, and functionally, into RPE-like cells. Based on these findings, differentiated ES cells have the potential for the development of new therapeutic approaches for RPE-specific diseases such as certain forms of retinitis pigmentosa and macular degeneration. Nevertheless, stringent control of retinal detachment and teratoma development will be necessary before initiation of treatment trials.

Keywords

Embryonic stem cell; Retinal pigment epithelium; RPE65; Retinal degeneration

The death of cells in the cell layer known as the retinal pigment epithelium (RPE) leads to blindness in many diseases, including age-related macular degeneration (AMD) and various forms of retinitis pigmentosa (RP). AMD alone affects approximately 8 million Americans, and its incidence is expected to double by 2020. Hence, RPE loss accounts for a significant number of neurodegenerative diseases that severely impair activities of daily living and cause psychological depression. Cell transplantation into the human retina has the potential to restore lost vision and to provide treatment of advanced stages of retinal degeneration with significant RPE loss (1–6). Replacement of damaged RPE in AMD is currently offered in many hospitals (7–16). However, donor tissue from primary fetal RPE and neuroretinal sheets are not readily obtainable from aborted fetuses, and endogenous viruses and other pathogens may not be easily detected in primary fetal cultures.

Stem cells, in contrast, may provide improved cell transplantation material for future clinical practice. Recent studies suggest that ES cell-derived RPE cells are more akin to *in vivo* RPE cells than human RPE cell lines in morphology, gene expression, and immunohistochemical analysis (17–19). Although some differentiation protocols have derived RPE from ES cells of rodent (4,20) and primate (1,6,21) origin, no studies have reported using these differentiated ES cells in treating diseased animals of the same species. It is imperative to have results from animal disease models before applying this type of treatment to humans.

Retinal pigment epithelium-specific protein 65 kDa (RPE65) is an all-*trans* to 11-*cis* isomerase (22). Thus, if RPE65 is nonfunctional or absent, then rhodopsin cannot be formed because no 11-*cis*-retinal is synthesized. Some inherited retinal diseases, including Leber congenital amaurosis (LCA) and RP, are caused by mutations in the gene encoding *RPE65*. An experimental mouse model, *Rpe65^{tm1}/Rpe65^{tm1}* (129/Sv strain background) (23), has been developed to monitor the efficacy of RPE transplantation. We have successfully rescued this mutant phenotype by using RPE transplants (24). An apparent limitation of the *Rpe65^{tm1}/Rpe65^{tm1}* model used in this original study was that RPE rescue persisted for only 4 months, most likely because of rejection of the RPE graft. The diseased mice were in the 129/Sv background, whereas the donor tissue was from mice of the C57BL/6J strain. Host rejection also limited other ES cell transplantation studies in the retina (25,26). However, rejection can be overcome by using isogenic donor cells and recipient animals.

More recently, a spontaneous mutant rodent model of *Rpe65* LCA, the *Rpe65^{rd12}/Rpe65^{rd12}* (*rd12*) mouse (C57BL/6J genetic background), has been reported (27). This mouse is of the same genetic background as our newly derived C2J ES cells, from a C57BL/6J-*Tyr^{c-2j}/J* mouse. In other words, our C2J donor cells should not be rejected by a C57BL/6J host. In addition, we have genetically engineered C2J cells to express a yellow fluorescent protein (YFP) to mark the location of transplants by live imaging.

The purpose of this study was to determine whether C2J ES cells will differentiate into RPE-like cells and improve retinal function in the *rd12* mouse. To accomplish this, we transplanted ES cell-derived RPE-like cells into the subretinal space of postnatal day 5 (P5) *rd12* mice. To determine whether any rescue effects were due to surgery or feeder cells, we grafted an additional three groups of *rd12* mice with phosphate-buffered saline (PBS), mitomycin-C treated PA6 feeders, and mitomycin-C treated undifferentiated mouse ES cells. Encouragingly, the ES cell-derived RPE-like cells expressed RPE markers, and the mice transplanted with these cells showed significant responses by electroretinogram (ERG) that did not occur in the control groups.

MATERIALS AND METHODS

Animals

Rpe65^{rd12}/Rpe65^{rd12} mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice were maintained in the Columbia University Pathogen-Free Eye Institute Annex Animal Care Services Facilities under a 12/12-hour light/dark cycle. All experiments were approved by the local Institutional Animal Care and Use Committee, and mice were used in accordance with the Statement for the Use of Animals in Ophthalmic and Vision Research of the Association for Research in Vision and Ophthalmology and the Policy for the Use of Animals in Neuroscience Research of the Society for Neuroscience.

Cell Culture

ES cells and PA6 cell lines were maintained as described (28). The methods used to induce undifferentiated mouse ES cells to differentiate into RPE-like cells were performed with modifications to previous protocols (4,20). After 7 days of in vitro differentiation, $1.0 \times 10^3/1 \mu\text{L}$ ES cell-derived RPE-like cells were transplanted into the subretinal space of postnatal day 5 (P5) *rd12* mice. To observe morphological changes and test the expression of RPE markers, ES cells were differentiated in vitro with the differentiation medium for longer periods of time (Fig. 1). Detailed differentiation protocols are provided in the Supplementary text.

Immunostaining for RPE Markers

After differentiation, cells cultured on cover slips were fixed and permeabilized with ice-cold methanol for 15 min at room temperature. Immunostaining was performed as described previously (29,30). Detailed procedures are provided in the Supplementary text.

Western Blot Analysis

After differentiation, cells were harvested for western blot analysis. Proteins were extracted from cell pellets and separated by 7.5% SDS polyacrylamide gel electrophoresis as described previously (29,31). More detailed methods, including antibody and titer, are available in the Supplementary text.

Transplantation of RPE-Like ES Cells

The RPE-like ES cells were injected into the subretinal space of P5 *rd12* mice as described previously (32). Detailed procedures and preparation of the cells are provided in the Supplementary text.

Live Imaging and Whole-Mount Eyecup Imaging

To assess transplantation efficiency, retinas were examined by live imaging as described previously (33,34). Whole-mount eyecups were prepared as described previously (30). Detailed procedures are provided in the Supplementary text.

Functional Measurement by ERG

ERG was conducted as described previously (32,35–39). Detailed procedures are provided in the Supplementary text.

Statistical Analysis

Statistical analyses were performed using SPSS software (SPSS, Chicago, IL). Unpaired *t* tests were used, and statistical significance was defined as $P < 0.05$.

RESULTS

Differentiation of Epithelial-Like Cells in Vitro

To determine whether in vitro differentiation could generate epithelial-like cells, ES cells were differentiated in the same media for longer periods of time than used for the cells intended for transplantation. Cultures examined at day 7 resembled overgrown ES colonies (Fig. 2A). After 7 days of differentiation, the cells at the margin of the colonies began to increase in size and continued to proliferate. Approximately 23 days of differentiation, some cells exhibited an epithelial morphology (Fig. 2B). On average, 30% to 50% of the colonies formed epithelial-like cells after 3 weeks of differentiation. We used C57BL/6J (B6) ES cells to assess whether this protocol can derive pigmented cells from ES cells, because C2J stem cells are albino. As shown in Figure 2(C), these B6 stem cells became pigmented and resembled RPE cells in morphology. These findings suggest that this protocol can derive pigmented epithelium from ES cells.

Immunocytochemistry and Immunoblots for RPE Markers

To explore whether the ES cells could assume an RPE cell fate after differentiation, we tested RPE markers including RPE65, bestrophin and ZO-1.

We performed immunoblots on extracts from the ES cells after 11 days of differentiation. To avoid possible contamination by PA6 cells, we replated the cells onto a freshly gelatinized 10-mm dish for 30 min. The PA6 cells then remained largely attached to the surface, and most cells left in suspension were differentiated ES cells. After 11 days of differentiation, ES cell-derived RPE-like cells contained RPE65 protein (Fig. 3A), whereas bestrophin protein expression remained low (Fig. 3A). Human adult and fetal RPE were used as positive controls in the immunoblot analysis. The corresponding molecular weights of RPE65 (65 kDa) and bestrophin (68 kDa) are indicated on the left-hand side of the figure.

To further confirm results from the immunoblot analysis, we performed immunocytochemical studies. Fourteen days after differentiation, RPE65 and ZO-1 were detected in the cytoplasm and the cell margin, respectively (Fig. 3B–E). The cytoplasmic distribution of RPE65 was more evenly distributed surrounding the nucleus at 28 days differentiation (Fig. 3F–I). These results indicate that ES cells could express some RPE markers after differentiation.

To further characterize the cell differentiation after transplantation, we performed immunohistochemical studies to demonstrate the subretinal location of B6 ES graft at 2 weeks after surgery (Fig. 4). Immunostaining with anti-RPE65 confirmed proper differentiation of grafted pigmented B6 cells and their subretinal location in an albino host.

Transplantation of ES Cells Into an *rd12* Mouse Model of RP

One hundred twenty-three mice received transplantations with ES cell-derived RPE-like cells. Seventy-six mice were excluded from the functional analysis by ERG because of total retinal detachment or tumor formation. Most mice with orbital tumor formation developed

the condition approximately 3 weeks after injection, and some mice presented with a subretinal mass or retinal detachment after 3 weeks. We screened the retina 1–2 months after injection. No mice developed ocular mass or tumors after 2 months. Some tumors contained tissue components resembling normal derivatives of all three germ layers.

Three control groups included four mice injected with PBS, five mice transplanted with mitomycin-C treated PA6 cells, and six mice transplanted with mitomycin-C treated undifferentiated ES cells. There was no retinal detachment and no tumor formation in the control mice. There were different numbers of subjects in each control group because of variance in litter sizes. The high rate of complications in groups transplanted with ES cell-derived RPE-like cells is related to the pluripotency of undifferentiated ES cells.

Live Imaging and Whole-Mount Eyecup Imaging

Before we transplanted ES cell-derived RPE-like cells, *in vivo* live fluorescence microscopy was used to evaluate how long the YFP-labeled ES cells can survive in the subretinal space. We followed up with three locations in the injected eye at different time points. These included the optic nerve (center of the retina), mid-peripheral retina, and peripheral retina. We found that the YFP-positive cells are visible at the mid-peripheral and peripheral retina at 9, 15, and 28 weeks after cell transplantation (Supplemental Fig. 1).

Whole-mount eyecup of a functional rescued eye transplanted with YFP-labeled ES cell-derived RPE-like cells revealed fluorescence-positive cells under the neurosensory retina (white oval in Fig. 5A). The distribution of the YFP-positive cells is around the equator of the eyeball, which corresponds to the injection site. A representative cryosection of the same mount showed YFP-positive cells (green color) between the outer segment and RPE (white arrow in Fig. 5B, nuclei were stained with DAPI). The gap between the outer segment and RPE layer is artificial detachment caused by the fixation process because there is no transudate in this gap. Results from both *in vivo* live imaging and whole-mount eyecup show that the transplanted cells survive and can be located between the outer segment and the native RPE layer for as long as 7 months after transplantation.

Restoration of ERG Activities After ES Cell-Derived RPE-Like Cell Transplantation

In total, 47 mice underwent ERG, and 12 mice showed increase ERG response in the transplanted eyes compared with the control fellow eyes. ERG results from transplanted mice are shown in Figure 6. Figure 6A shows recordings from mice at 3 months after being transplanted with ES cell-derived RPE-like cells. Figure 6B displays maximal ERG b-wave enhancement from 1 to 7 months posttransplantation. b-wave enhancement is defined as the difference in maximum ERG responses of transplanted and control fellow eyes (μV). Significant differences in the b-waves generated in the control and transplanted eyes were not detected at 1 or 2 months after transplantation (black solid bars in Fig. 6B) but were observed at 3 and 6 months posttransplantation. At 3 and 6 months posttransplantation, ERGs from transplanted eyes show a statistically significant rescue effect ($P = 0.001$ and $P = 0.038$, respectively) (black solid bars in Fig. 6B). Although the difference was not statistically significant at 4, 5, and 7 months after transplantation, the b-wave amplitudes in the transplanted eyes were consistently higher than the control fellow eyes (black solid bars in Fig. 6B).

To confirm that this effect is not caused by surgical injury response nor by trophic factors released from ES cells, we also tested mice injected with PBS, mitomycin-C treated PA6 feeder cells, and mitomycin-C treated undifferentiated ES cells. The ERGs from these mice did not reveal any statistical significant difference between injected eyes and noninjected eyes (white, light shaded, and dark shaded bars in Fig. 6B). The results from both

experimental groups and control groups indicate that the rescue effect in our experimental groups is from these ES cell-derived RPE-like cells and not from PBS, PA6 feeder cells, or undifferentiated ES cells.

DISCUSSION

This study provides the first evidence that ES cell-derived RPE-like cells restore visual function in a clinically relevant mouse model of retinal disease. Stem-cell derivatives can be genetically marked by expression of fluorescent proteins and tracked in live animals by noninvasive imaging. In vitro cultures showed that the PA6 stromal cell line can induce differentiated mouse embryonic stem (ES) cells to express RPE-specific markers, such as RPE65, bestrophin, and ZO-1. Transplanted stem cells express RPE65 isomerase, restore vitamin A metabolism nonautonomously, and halt retinal degeneration. We showed efficacy of stem-cell transplantation by ERGs. ERGs demonstrate that retinal function can be restored and indicate that stem-cell derivatives integrate functionally into degenerating *rd12* retina. We conclude that stem-cell transplantation has the potential to restore lost vision and provide treatment of advanced stages of RP and AMD featuring significant RPE loss.

Differentiating ES Cells Into RPE-Like Cells

The expression of RPE-specific markers in cells resulting from in vitro differentiation of ES cells correlates with the differentiation states of RPE cells in utero (17). In addition, bestrophin has been previously identified as a late marker of RPE differentiation (40–42). In this study, immunoblot analyses demonstrated that the differentiated ES cells expressed RPE65 earlier than they expressed bestrophin. Because RPE65 is expressed in endoplasmic reticulum, the pattern of RPE65 from day 14 to day 28 could be explained by an increase in flattened cells at day 28 compared with day 14, which means that the cells became more epithelial-like after longer in vitro differentiation. The differentiated ES cells have the potential to express RPE65 in vitro, and they might be able to express these RPE markers in vivo in their native environment in the developing mouse retina.

There are several differentiation protocols to derive RPE from ES cells, including rodent ES cells (4,20) and primate ES cells (1,6,21). Our study used the protocols for rodent ES cells, which characterized differentiation in morphology, gene expression, and immunohistochemical studies (4,20). Recently, Osakada et al. (19) reported in vitro differentiation methods for mouse, monkey, and human ES cells under defined conditions. These feeder- and serum-free conditions will form the basis for human ES cell-based therapies.

Even with the recent differentiation protocol, approximately 25% of the differentiated colonies express RPE markers at day 40 of differentiation (41). Although our differentiation protocol and ES cell species are different from this article, the results from this article may explain the unsatisfied rescue rate (25.5%) in our preliminary study.

Survival of Donor Cells After Transplantation

Noninvasive and nondestructive imaging of live mice is an efficient assay that enables us to monitor fluorescently-labeled transplanted stem cells. Long-term survival of grafted cells can be assessed with live imaging. YFP expression marks donor ES cells after subretinal transplantation, allowing stem-cell progeny to be distinguished from host cells in live animals.

Functional Analysis of Mice Transplanted With RPE-Like Cells

Both *rd12* and *Rpe65^{tm1}/Rpe65^{tm1}* mice have early-onset blindness that is demonstrated by profoundly reduced activity by ERG (24,27). These mice lack RPE65 isomerase activity, 11-*cis* retinal, and proper vitamin A metabolism. In previous studies, we successfully rescued the *Rpe65^{tm1}/Rpe65^{tm1}* mice by RPE transplantation, and we found that these grafted RPE cells do not need contact with Bruch's membrane to restore retinal function (24). Because a few transplanted cells can restore RPE65 isomerase activity and vitamin A metabolism nonautonomously, global visual function can be improved (24).

The Royal College of Surgeons (RCS) rat, different from *rd12* mice, has a mutation of the receptor tyrosine kinase *merlk* gene, which precludes RPE cells from phagocytosis and leads to the accumulation of debris in the subretinal space and ultimately to the death of photoreceptors before 3 months of age (43). Previous studies reported functional rescue in RCS rats transplanted with either the human RPE cell line (ARPE-19) (44,45) or the RPE cells derived from human ES cells (18,42). However, the functional rescue in the RCS rats can be explained by the disruption and washing of debris in the subretinal space, because local areas of functional rescue can be observed in the RCS rats up to 6 months after sham injections (46).

To avoid similar phenomena in our study, we included three control groups representing injections with PBS, mitomycin-C treated PA6 feeders, and mitomycin-C treated undifferentiated mouse ES cells. All the mice in the same experimental group were litter mates, and the time points for transplantation and ERG were all the same. Therefore, to avoid variability between different groups and different litters, we compared the ERGs from transplanted and control fellow eyes in the same groups. The ERGs from mice in the three control groups showed no statistically significant differences between transplanted and control fellow eyes, whereas the ERGs from mice transplanted with ES cell-derived RPE-like cells showed statistically significant differences between transplanted and control fellow eyes. These results implied that the differentiated ES cells had the ability to restore visual function.

We used mitomycin-C treated undifferentiated ES cells as a control because nonmitomycin-C treated undifferentiated ES cells caused teratoma development within 3 weeks after transplantation. After treating these undifferentiated ES cells with mitomycin-C, no mice developed teratomas after transplantation. This indicates that our YFP-labeled C2J ES cells are pluripotent, and this potency of proliferation can be decreased by pretreatment with mitomycin-C.

Because the full-field ERG reflects a summation of activity from the whole eye and because the magnitude of the b-wave is proportional to the area and degree of visual field preservation (47), it is reasonable to assume that the small gains in the amplitude of the b-waves observed here might be due to the preservation of only a focal part of the retina by transplanted cells (Fig. 5B). Thus, insufficient amounts of preserved retina may lead to an inability to observe ERG amplitude gains in some eyes transplanted with ES cell-derived RPE-like cells. There is a limitation in our experiments, in that we are not able to determine how many cells remained in the subretinal space despite the fact that we counted the cells before transplantation. It is reasonable to assume that if we transplanted more RPE-like cells, then larger areas of the retina would be restored by RPE65 function. However, increasing the numbers of transplanted cells may increase the risk of ocular tumor if the differentiation protocol fails to differentiate all ES cells into RPE-like cells, and these undifferentiated ES cells are carried along through transplantation.

Tumor formation is a concern when applying ES cell-based therapy to treat degenerative diseases (48,49), and it is rarely seen in previous retinal transplantations using nonisogenic ES donors (18,42). Tumors develop after transplantation because pluripotent ES cells remain after the differentiation protocol is conducted. Because syngeneic ES cell transplantation serves as a model for the clinical use of induced pluripotent stem cells (iPS) in patients, both ES- and iPS-based regenerative medicinal approaches face similar challenges in the risk of tumor development. The efficacy and outcome of ES and iPS-based therapies can be improved by engineering these cells with either genes encoding fluorescent proteins or a resistance to antibiotics driven by RPE-specific promoters. Then, the terminally differentiated cells can be selected. In this way, the exclusion of residual undifferentiated cells should eliminate teratoma or tumor development. Furthermore, the ideal differentiation protocol will result in minimal undifferentiated cells and derive RPE under feeder- or serum-free conditions. Because the differentiation pathways followed by human induced pluripotent stem (iPS) cells and mouse ES cells are remarkably similar (50–53), RPE cells derived from iPS cells are a potentially unlimited source of RPE.

Enabling the Use of Stem-Cell Therapy in Future Clinical Trials

The inability to maintain life-long functioning stem-cell graft represents a barrier before the initiation of human studies. Local immunosuppression of the eye with cyclosporine will hence be necessary to maintain graft survival (54). Alternatively, the gene-targeting technologies that are already established in ES cells can make them more readily immunotolerant than primary fetal cells. For example, host-specific major histocompatibility complex antigens could be expressed in ES cells to prevent their rejection by the host.

With respect to future clinical practice, the use of validated universal stem-cell lines is more likely to accomplish “Good Manufacturing Practice Compliance” than the use of either heterogeneous primary fetal RPE or neuroretinal sheets from various donors. In fact, primary fetal RPE and neuroretinal sheets from aborted fetuses are not readily obtainable.

In summary, our studies indicate that PA6-treated ES cells express multiple RPE-specific markers and provide evidence that ES cells have the potential to differentiate morphologically and functionally into RPE. More than half of the mice were complicated with retinal detachments or tumor development, although one fourth of the mice showed increased ERG responses in the transplanted eyes. These transplanted RPE-like cells can restore neuronal function in the *rd12* mouse model over a 7-month period, as shown by ERG recordings. Thus, transplantation of stem cells may represent a future option in treating some RPE-specific diseases, such as certain forms of RP and macular degeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Haruta M, Sasai Y, Kawasaki H, et al. In vitro and in vivo characterization of pigment epithelial cells differentiated from primate embryonic stem cells. *Invest Ophthalmol Vis Sci* 2004;45:1020. [PubMed: 14985325]
2. Klassen H, Sakaguchi DS, Young MJ. Stem cells and retinal repair. *Prog Retin Eye Res* 2004;23:149. [PubMed: 15094129]
3. Haruta M. Embryonic stem cells: Potential source for ocular repair. *Semin Ophthalmol* 2005;20:17. [PubMed: 15804840]
4. Aoki H, Hara A, Nakagawa S, et al. Embryonic stem cells that differentiate into RPE cell precursors in vitro develop into RPE cell monolayers in vivo. *Exp Eye Res* 2006;82:265. [PubMed: 16150443]
5. Banin E, Obolensky A, Idelson M, et al. Retinal incorporation and differentiation of neural precursors derived from human embryonic stem cells. *Stem Cells* 2006;24:246. [PubMed: 16123388]
6. Lamba DA, Karl MO, Ware CB, et al. Efficient generation of retinal progenitor cells from human embryonic stem cells. *Proc Natl Acad Sci USA* 2006;103:12769. [PubMed: 16908856]
7. Binder S, Stanzel BV, Krebs I, et al. Transplantation of the RPE in AMD. *Prog Retin Eye Res* 2007;26:516. [PubMed: 17532250]
8. MacLaren RE, Uppal GS, Balaggan KS, et al. Autologous transplantation of the retinal pigment epithelium and choroid in the treatment of neovascular age-related macular degeneration. *Ophthalmology* 2007;114:561. [PubMed: 17324698]
9. Algrever PV, Gouras P, Dafgard Kopp E. Long-term outcome of RPE allografts in non-immunosuppressed patients with AMD. *Eur J Ophthalmol* 1999;9:217. [PubMed: 10544978]
10. da Cruz L, Chen FK, Ahmado A, et al. RPE transplantation and its role in retinal disease. *Prog Retin Eye Res* 2007;26:598. [PubMed: 17920328]
11. Tezel TH, Del Priore LV, Berger AS, et al. Adult retinal pigment epithelial transplantation in exudative age-related macular degeneration. *Am J Ophthalmol* 2007;143:584. [PubMed: 17303061]
12. Del Priore LV, Tezel TH, Kaplan HJ. Maculoplasty for age-related macular degeneration: Reengineering Bruch's membrane and the human macula. *Prog Retin Eye Res* 2006;25:539. [PubMed: 17071125]
13. Jousseaume AM, Joeres S, Fawzy N, et al. Autologous translocation of the choroid and retinal pigment epithelium in patients with geographic atrophy. *Ophthalmology* 2007;114:551. [PubMed: 17324697]
14. Kaplan HJ, Tezel TH, Del Priore LV. Retinal pigment epithelial transplantation in age-related macular degeneration. *Retina* 1998;18:99. [PubMed: 9564688]
15. Jousseaume AM. Cell transplantation in age related macular degeneration: Current concepts and future hopes. *Graefes Arch Clin Exp Ophthalmol* 2004;42:1. [PubMed: 14745559]
16. Thomas BB, Aramant RB, Sadda SR, et al. Retinal transplantation. A treatment strategy for retinal degenerative diseases. *Adv Exp Med Biol* 2006;572:367. [PubMed: 17249598]
17. Klimanskaya I, Hipp J, Rezai KA, et al. Derivation and comparative assessment of retinal pigment epithelium from human embryonic stem cells using transcriptomics. *Cloning Stem Cells* 2004;6:217. [PubMed: 15671670]
18. Lund RD, Wang S, Klimanskaya I, et al. Human embryonic stem cell-derived cells rescue visual function in dystrophic RCS rats. *Cloning Stem Cells* 2006;8:189. [PubMed: 17009895]
19. Osakada F, Ikeda H, Mandai M, et al. Toward the generation of rod and cone photoreceptors from mouse, monkey and human embryonic stem cells. *Nat Biotechnol* 2008;26:215. [PubMed: 18246062]

20. Hirano M, Yamamoto A, Yoshimura N, et al. Generation of structures formed by lens and retinal cells differentiating from embryonic stem cells. *Dev Dyn* 2003;228:664. [PubMed: 14648843]
21. Carr AJ, Vugler A, Lawrence J, et al. Molecular characterization and functional analysis of phagocytosis by human embryonic stem cell-derived RPE cells using a novel human retinal assay. *Mol Vis* 2009;15:283. [PubMed: 19204785]
22. Jin M, Li S, Moghrabi WN, et al. Rpe65 is the retinoid isomerase in bovine retinal pigment epithelium. *Cell* 2005;122:449. [PubMed: 16096063]
23. Redmond TM, Yu S, Lee E, et al. Rpe65 is necessary for production of 11-cis-vitamin A in the retinal visual cycle. *Nat Genet* 1998;20:344. [PubMed: 9843205]
24. Gouras P, Kong J, Tsang SH. Retinal degeneration and RPE transplantation in Rpe65(−/−) mice. *Invest Ophthalmol Vis Sci* 2002;43:3307. [PubMed: 12356839]
25. Schraermeyer U, Thumann G, Luther T, et al. Subretinally transplanted embryonic stem cells rescue photoreceptor cells from degeneration in the RCS rats. *Cell Transplant* 2001;10:673. [PubMed: 11814109]
26. Meyer JS, Katz ML, Maruniak JA, et al. Neural differentiation of mouse embryonic stem cells in vitro and after transplantation into eyes of mutant mice with rapid retinal degeneration. *Brain Res* 2004;1014:131. [PubMed: 15212999]
27. Pang JJ, Chang B, Hawes NL, et al. Retinal degeneration 12 (rd12): A new, spontaneously arising mouse model for human Leber congenital amaurosis (LCA). *Mol Vis* 2005;11:152. [PubMed: 15765048]
28. Yamane T, Hayashi S, Mizoguchi M, et al. Derivation of melanocytes from embryonic stem cells in culture. *Dev Dyn* 1999;216:450. [PubMed: 10633864]
29. Tosi J, Janisch KM, Wang NK, et al. Cellular and molecular origin of circumpapillary dysgenesis of the pigment epithelium. *Ophthalmology* 2009;116:971. [PubMed: 19410955]
30. Wang NK, Fine HF, Chang S, et al. Cellular origin of fundus autofluorescence in patients and mice with a defective NR2E3 gene. *Br J Ophthalmol* 2009;93:1234. [PubMed: 19429590]
31. Hamel CP, Tsilou E, Harris E, et al. A developmentally regulated microsomal protein specific for the pigment epithelium of the vertebrate retina. *J Neurosci Res* 1993;34:414. [PubMed: 8474143]
32. Davis RJ, Tosi J, Janisch KM, et al. Functional rescue of degenerating photoreceptors in mice homozygous for a hypomorphic cGMP phosphodiesterase 6 b allele (Pde6bH620Q). *Invest Ophthalmol Vis Sci* 2008;49:5067. [PubMed: 18658088]
33. Nagasaki T, Zhao J. Centripetal movement of corneal epithelial cells in the normal adult mouse. *Invest Ophthalmol Vis Sci* 2003;44:558. [PubMed: 12556383]
34. Nagasaki T, Zhao J. Uniform distribution of epithelial stem cells in the bulbar conjunctiva. *Invest Ophthalmol Vis Sci* 2005;46:126. [PubMed: 15623764]
35. Salchow DJ, Gouras P, Doi K, et al. A point mutation (W70A) in the rod PDE-gamma gene desensitizing and delaying murine rod photoreceptors. *Invest Ophthalmol Vis Sci* 1999;40:3262. [PubMed: 10586951]
36. Tsang SH, Burns ME, Calvert PD, et al. Role of the target enzyme in deactivation of photoreceptor g protein in vivo. *Science* 1998;282:117. [PubMed: 9756475]
37. Tsang SH, Gouras P, Yamashita CK, et al. Retinal degeneration in mice lacking the g subunit of rod cGMP phosphodiesterase. *Science* 1996;272:1026. [PubMed: 8638127]
38. Tsang SH, Woodruff ML, Jun L, et al. Transgenic mice carrying the H258N mutation in the gene encoding the beta-subunit of phosphodiesterase-6 (PDE6B) provide a model for human congenital stationary night blindness. *Hum Mutat* 2007;28:243. [PubMed: 17044014]
39. Tsang SH, Chen J, Kjeldbye H, et al. Retarding photoreceptor degeneration in Pdegtm1/Pdegtm1 mice by an apoptosis suppressor gene. *Invest Ophthalmol Vis Sci* 1997;38:943. [PubMed: 9112990]
40. Bakall B, Marmorstein LY, Hoppe G, et al. Expression and localization of bestrophin during normal mouse development. *Invest Ophthalmol Vis Sci* 2003;44:3622. [PubMed: 12882816]
41. Meyer JS, Shearer RL, Capowski EE, et al. Modeling early retinal development with human embryonic and induced pluripotent stem cells. *Proc Natl Acad Sci USA* 2009;106:16698. [PubMed: 19706890]

42. Idelson M, Alper R, Obolensky A, et al. Directed differentiation of human embryonic stem cells into functional retinal pigment epithelium cells. *Cell Stem Cell* 2009;5:396. [PubMed: 19796620]
43. D'Cruz PM, Yasumura D, Weir J, et al. Mutation of the receptor tyrosine kinase gene *Mertk* in the retinal dystrophic RCS rat. *Hum Mol Genet* 2000;9:645. [PubMed: 10699188]
44. Sauve Y, Pinilla I, Lund RD. Partial preservation of rod and cone ERG function following subretinal injection of ARPE-19 cells in RCS rats. *Vision Res* 2006;46:1459. [PubMed: 16364396]
45. Wang S, Lu B, Girman S, et al. Morphological and functional rescue in RCS rats after RPE cell line transplantation at a later stage of degeneration. *Invest Ophthalmol Vis Sci* 2008;49:416. [PubMed: 18172120]
46. Gaillard F, Sauve Y. Cell-based therapy for retina degeneration: The promise of a cure. *Vision Res* 2007;47:2815. [PubMed: 17719072]
47. Sauve Y, Lu B, Lund RD. The relationship between full field electroretinogram and perimetry-like visual thresholds in RCS rats during photoreceptor degeneration and rescue by cell transplants. *Vision Res* 2004;44:9. [PubMed: 14599567]
48. Aoki H, Hara A, Niwa M, et al. Transplantation of cells from eye-like structures differentiated from embryonic stem cells in vitro and in vivo regeneration of retinal ganglion-like cells. *Graefes Arch Clin Exp Ophthalmol* 2008;246:255. [PubMed: 18004585]
49. Chaudhry GR, Fecek CM, Lia M, et al. Fate of embryonic stem cell derivatives implanted into the vitreous of a slow retinal degenerative mouse model. *Stem Cells Dev* 2009;18:247. [PubMed: 18442304]
50. Takahashi K, Tanabe K, Ohnuki M, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007;131:861. [PubMed: 18035408]
51. Park IH, Zhao R, West JA, et al. Reprogramming of human somatic cells to pluripotency with defined factors. *Nature* 2008;451:141. [PubMed: 18157115]
52. Pera MF. Stem cells. A new year and a new era. *Nature* 2008;451:135. [PubMed: 18185576]
53. Yamanaka S. Induction of pluripotent stem cells from mouse fibroblasts by four transcription factors. *Cell Prolif* 2008;41:51. [PubMed: 18181945]
54. Lai CC, Gouras P, Doi K, et al. Local immunosuppression prolongs survival of RPE xenografts labeled by retroviral gene transfer. *Invest Ophthalmol Vis Sci* 2000;41:3134. [PubMed: 10967075]

FIGURE 1.

Schematic overview of experimental settings yellow fluorescent protein (YFP)-labeled C2J embryonic stem (ES) cells were cocultured on previously seeded mitomycin C-treated PA6 cells in differentiation medium (A). After 7 days of differentiation, ES cells were continuously differentiated to observe the morphological change and tested for retinal pigment epithelial (RPE) markers by immunocytochemistry staining and western blot analysis (B). After 7 days of differentiation, ES cells were collected and transplanted into the subretinal space of postnatal day 5 (P5) *Rpe65^{rd12}/Rpe65^{rd12}* mice. These mice were followed by in vivo live imaging and functionally analyzed by ERG (C).

FIGURE 2.

(A) Epithelial morphology of differentiated embryonic stem (ES) cells. Differentiation of yellow fluorescent protein (YFP)-labeled C2J ES cells at day 7 on PA6 feeders. (B) After 23 days in vitro, some YFP-labeled C2J ES cell colonies contained epithelial-like cells. (C) Some B6 ES cells were pigmented after 64 days. Scale bar denotes 100 µm.

FIGURE 3.

Retinal pigment epithelial (RPE) immunoreactivity is found in cultured embryonic stem (ES) cells as they differentiate. (A) Immunoblots of ES-derived RPE extract after 11 days of differentiation probed with anti-RPE65 and anti-bestrophin antibodies. Human adult RPE and fetal RPE extracts were used as positive controls. Molecular weights of RPE65 and bestrophin are indicated on the left (B–M). Immunocytochemical staining of the RPE-like cells after 14 and 28 days differentiation expressed ZO-1 at the cell margin (*green*, B and F) and RPE65 in the cytoplasm (*purple*, C and G). Nucleus is stained with DAPI (*cyan*, D, H, and L). Negative control at 14 days differentiation (J–M) shows only nucleus staining. Scale bars denote 25 μm.

FIGURE 4.

Retinal pigment epithelium-specific protein 65 kDa (RPE65) expression in the B6 embryonic stem (ES) cell-derived RPE graft in retinal sections of albino mice at 2-week posttransplantation. Immunostaining of retinal sections of BALB/cJ (albino) mice with anti-RPE65 antibody are shown (63 $\times$). PBS injected control retina with monolayer of RPE (A); B6 ES cell-derived RPE grafts in the subretinal space of an albino *Pde6g^{tm1}/Pde6g^{tm1}* mouse (B–D). *Pde6g^{tm1}/Pde6g^{tm1}* mice lack photoreceptor outer segments. Note the transplanted cells overlaying host RPE cells (*red arrows*). Melanin granules can be seen in the B6 transplant graft (C). Notice double layer splitting after inner retina was mechanically pulled to delineate transplanted B6 ES cells (C). *Red arrows* mark RPE cells.

FIGURE 5.

Long-term survival and distribution of embryonic stem (ES) transplants. (A) Whole-mount retina of a mouse grafted with yellow fluorescent protein (YFP)-labeled ES cell-derived retinal pigment epithelial (RPE)-like cells; YFP-positive cells around the injection site (*white oval*). (B) Histochemical analysis of representative sections from the whole-mount retina shown in (A) revealed an YFP-positive cell located between the OS and the RPE. IS/OS, inner segment and outer segment; ONL, outer nuclear layer; INL, inner nuclear layer.

FIGURE 6.

ERGs of *Rpe65^{rd12}/Rpe65^{rd12}* mice after subretinal transplantation with ES cell-derived retinal pigment epithelial (RPE)-like cells confirm functional rescue. (A) ERG from mice after 3 months transplantation. Eyes transplanted with ES cell-derived RPE-like cells (upper) showed higher b-wave amplitudes compared with control fellow eyes (lower). Traces represent readings from different mice. (B) b-wave enhancement in mice 1 to 7 months posttransplantation, as indicated by black solid bars. b-wave enhancement is defined as the difference in maximum ERG responses of transplanted and control fellow eyes (μV). Unpaired *t* tests were performed for paired differences in b-wave peaks between transplanted and control eyes. At 3 and 6 months posttransplantation, ERGs from transplanted eyes show a statistically significant rescue effect (***P* = 0.001 and **P* = 0.038, respectively). Although the difference was not statistically significant at 4, 5, and 7 months after transplantation, the b-wave amplitudes in the transplanted eyes were consistently higher than the control fellow eyes. The number of mice analyzed per time point is indicated. ERGs were performed on both eyes (injected and control) simultaneously. There is no statistically significant difference between injected and control eyes in the other three control groups. White bar, b-wave enhancement in PBS injected mice; light-shaded bar, b-wave enhancement in mitomycin-C treated PA6 cell transplanted mice; and dark-shaded bar, b-wave enhancement in mitomycin-C treated undifferentiated ES cell transplanted mice. ES-RPE: ES cell-derived RPE-like cells, Mit-C, mitomycin-C; ES, embryonic stem; PBS, phosphate buffered saline.