Retinal Degeneration and RPE Transplantation in Rpe65$^{-/-}$ Mice

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Purpose. To determine whether transplanting normal retinal pigment epithelium (RPE) into the subretinal space influences photoreceptor function and degeneration in Rpe65$^{-/-}$ mice.

Methods. RPE cells were isolated from eyes of normal mice and transplanted to the subretinal space of one eye of Rpe65$^{-/-}$ mice. The other eye received a subretinal injection of saline or was not touched. Corneal electroretinograms (ERGs) from both eyes were monitored before and after surgery to follow progression of the degeneration. The width of the outer nuclear layer was measured in the area of transplantation and compared with a similar area in control retinas.

Results. Transplantation of RPE increased ERG amplitude maximally at 3.7 weeks after surgery. This rescue effect slowly diminished with time. Sham surgery had little effect on the ERG. The width of the outer nuclear layer in the area receiving RPE transplants was slightly greater than in control subjects. Evidence of the presence of RPE transplants in the subretinal space decreased with time after transplantation without signs of inflammation.

Conclusions. Retinal degeneration in the Rpe65$^{-/-}$ mice is slowly progressive. Photoreceptor function can be transiently increased for several months and anatomic degeneration slightly reduced in Rpe65$^{-/-}$ mice by RPE cell transplantation. Loss of the rescue effect may be due to degeneration of the transplanted RPE. (*Invest Ophthalmol Vis Sci.* 2002;43: 3307–3311)

Several different gene defects are responsible for childhood retinal degeneration Leber's congenital amaurosis (LCA). One such defect prevents the RPE from synthesizing the 11-*cis* retinoids essential for vision.[1–8] This defect alters a protein Rpe65[9,10] that is involved in the isomerization of all-*trans* to 11-*cis* retinol in the RPE cell.[11] There are two animal models of this disease. One has been produced in mice by targeted gene disruption, Rpe65$^{-/-}$.[11] The other is due to a naturally occurring 4-bp deletion in the same gene discovered in certain Briard dogs.[12–14] Oral administration of 9-*cis* retinal[15] can increase visual function in Rpe65$^{-/-}$ mice. Gene therapy also restored visual function in affected Briard dogs.[16] In this study we examined how transplantation of normal RPE to the subretinal space affects photoreceptor function and retinal degeneration.

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Methods

Animals

Rpe65$^{-/-}$ mice were obtained from breeding pairs generously provided by Michael Redmond (National Institutes of Health, Bethesda, MD). Nine litters totaling 36 Rpe65$^{-/-}$ mice received RPE transplants in one eye, whereas the other eye received no surgery or a subretinal injection of saline (Table 1). Seven litters were observed until they were 8 to 16 months of age, at which time they were killed for histology. One litter each was killed at 1, 4, and 6 months of age and another at 10 to 14 days after surgery to examine the transplanted RPE at shorter times after transplantation. Seven mice underwent surgery but were discarded because of unsatisfactory transplantation. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

RPE Transplantation

RPE cells for transplantation were obtained from normal C57BL/6 mice. After the death of the mice, the eyes were removed and kept at 4°C for 3 hours in Hank's balanced salt solution (HBSS; pH 7.4), to facilitate separation of the neural retina from the RPE layer. The anterior segment and neural retina were removed and the eyecup incubated in a 0.2% trypsin solution at pH 8.0 at 37°C for 45 minutes. Trypsinization was stopped with HBSS containing bovine serum and the eyecup washed with HBSS to remove the serum. Sheets of RPE cells were removed from Bruch's membrane with microforceps with the aid of a dissecting microscopic. Sheets and single hexagonal RPE cells were sucked up with a pipette, collected in a tube and centrifuged at 600 rpm for 5 minutes. The supernatant was removed and the bolus of cells triturated. An aliquot was used to determine the concentration of cells using a hemocytometer and identify contaminants. These solutions contain approximately 50,000 to 75,000 RPE cells in 100 μL. Some contamination with outer segment material and erythrocytes were occasionally seen. Choroidal melanocytes were rarely seen. The solution was kept on ice while recipient Rpe65$^{-/-}$ mice were anesthetized with ketamine (5 mg/mL) and xylazine (2 mg/kg) administered intraperitoneally. The pupils were dilated with 1% phenylephrine and 1% cyclopentolate. Each mouse was placed on its side with one eye facing a surgical microscope. An incision was made in the conjunctiva along the horizontal meridian at the temporal side of the eye. A scleral incision was made and the choroid identified using 40× magnification. A glass pipette with a tip diameter of 0.07 to 0.1 mm connected to a polyethylene tube and a syringe (Hamilton, Reno, NV) were used to draw up approximately 10 microliters of the transplant solution. A small air bubble separated this pigmented solution from the clear saline. The tip was introduced through the scleral incision and guided tangentially through the choroid to the subretinal space, where the cell solution was slowly injected. The mouse was rotated so that the pupil faced the microscope to examine the detachment of the neural retina through a thin glass coverslip on the cornea. A mouse was discarded if we did not detect a detachment or if there was indication of a break in the neural retina causing the cells to enter the vitreous. The other eye was either not touched, or a saline-induced detachment was produced in a corresponding area of the retina. The incisions in the sclera and conjunctiva were sutured with 10-0 nylon. After surgery, each mouse was warmed on a heating stage before being returned to its cage.

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TABLE 1. Data on Mice in the Study

Litters	Mice	Age at Surgery (m)	Transplants/ Rejects	Sham/ Untouched	Histology (m)
1	4	1.5	1/3	0/1	12
2	4	1.0	2/2	0/2	12
3	4	2.1	4/0	1/3	8, 12, 16
4	7	4.0	7/0	2/5	12, 16
5	5	1.0	5/0	2/3	1, 4, 6
6	6	2.0	6/0	3/3	10, 14*
7	5	3.7	5/0	0/5	8, 12, 16
8	4	1.9	4/0	0/4	16
9	4	2.6	2/2	0/2	16
Total	43	—	36/7	8/28	—

* Days.

ERG Recordings

Corneal electroretinograms (ERGs) were obtained from both eyes of each mouse weekly for 1 month or longer before surgery. After surgery, ERGs were obtained every 2 to 4 weeks until the mice were killed. The mice were dark adapted overnight and anesthetized under red light with ketamine and xylazine, as described, and then the pupils were dilated. ERGs were recorded from the cornea with a cotton wick saline electrode. Subcutaneous 30-gauge needles inserted into the forehead and trunk were reference and ground electrodes, respectively. The mouse rested on a heater that maintained body temperature at 35°C to 36°C. The light stimulus was obtained from a stroboscope (PS33; Grass Instruments, Inc., West Warwick, RI) removed from its housing and placed in metal case with a circular aperture 30 cm in diameter. The aperture was placed 20 cm from the cornea. Maximum flash intensity, measured at the cornea with a calibrated light meter (J16; Tektronix Instruments, Beaverton, OR) was 0.7×103 $\mu W/cm^2$. Filters were used to reduce the energy of the light flash. Responses were averaged by a computerized data acquisition system (Power Laboratory; AD Instruments, Mountain View, CA) at a frequency of 0.1 Hz, starting with the weakest and then progressing to stronger flashes. ERG amplitudes were measured from the initial negative peak of the a-wave or from the baseline to the positive peak of the b-wave.

Histology

After the death of the mice, the eyes were removed and the superior limbus pierced with a needle at 12 o'clock to establish the eye's polarity. The eyes were fixed in 4% glutaraldehyde in phosphate-buffered saline and kept at 4°C for 3 to 4 days before being washed and dissected. The anterior segment and lens were removed and the posterior pole cut with a razor blade along the horizontal meridian just below the optic nerve. The upper half of the posterior segment containing the optic nerve was dehydrated and embedded separately from the lower half. After Epon embedding, the block was cut along the horizontal meridian from the anterior retina toward the optic disc. Blocks were readjusted during cutting to keep sections of the retina as radial as possible. Sections, 1 to 2 μm in thickness and extending from anterior to posterior ora serrata, were examined every 150 μm. The width of the outer nuclear layer was measured every 0.2 mm in five to seven successive sections through the optic nerve head to cover the area that had received the transplanted cells. Corresponding areas were measured in control eyes. The widths at similar areas in each section were averaged. Significant differences between measurements were determined using the Student's *t*-test.

RESULTS

Figure 1 shows ERG amplitudes of test and control eyes of four litters of Rpe65$^{-/-}$ mice before and after RPE cell transplantation. Transplantation surgery occurred when the mice were between 1 and 5 months of age. In all cases, the average amplitude of the ERG of the eyes receiving transplants increased after surgery compared with control eyes. This difference diminished with time. Another three litters that received transplants and were observed for a similar period showed a similar pattern, although the ones selected for Figure 1 were the most significant. The maximum increase in ERG amplitude after transplantation, averaged over all seven litters, occurred at 3.7 ± 2.6 (SD) weeks after surgery. The average amplitude of this maximum ERG of mice receiving transplants was 84 ± 23 μV compared with 39 ±7 μV for untouched and 43 ± 5 μV for sham control subjects (significant at $P = 0.003$).

Figure 2 illustrates ERGs from an eye that had received a transplant 3 weeks previously, compared with the control eye. Both the amplitude and sensitivity of the ERG from the eye receiving the transplant was greater than that of the control eye.

Figure 3 illustrates the average ERG amplitudes in relation to the age of the mice. The time that surgery occurred is indicated by arrows. When the mice were grouped according to age, the overall amplitude of the ERG was consistently larger in those eyes receiving RPE transplants than in control subjects that underwent no surgery or saline injection. These results reveal a gradual decrease in the ERG amplitude of all eyes until approximately age 6 months, when a plateau in this relationship seemed to occur.

Histology

Figure 4 compares the width of the outer nuclear layer in the area of transplantation with a similar area in control retinas, superior to the optic nerve head toward the posterior side of the retina. There was a progressive decrease in the width of this layer in both control and test retinas. At 16 months of age, the width of the outer nuclear layer was approximately half that at 1 month of age. There is a suggestion that transplantation slows the rate of the degeneration of the outer nuclear layer, which is most significant ($P = 0.04$) at 1 year and insignificant at 16 months after transplantation.

Figure 5 shows the appearance of the photoreceptor outer segments at 6 months (Fig. 5A) and 1 year (Fig. 5B) of age in control mice. The outer segments had degenerated much more in the older mice, although occasionally more intact ones (Fig. 5B, large arrow) were found. Vacuoles (Fig. 5B, lower right) were prominent in the RPE layer at 1 year of age, presumably due to the increased ester content of these cells.[11]

Transplanted RPE was found in the retinas of all six eyes of mice killed at within 10 to 14 days after surgery but were more difficult to find at longer times. Figure 6 shows an example of what is considered to be an RPE transplant in the subretinal space at 10 days after surgery. In this sample, a sheet of transplanted cells had not completely dissociated. The transplanted cells contained normal amounts of melanin pigment and appeared similar to the host RPE. Such RPE cells were not found within the subretinal space in normal or sham-treated mice. Surgery through the choroid could produce displacement of RPE but this was limited to the area of the scar. All the cells considered to be transplants were far from the scarred area produced by the injecting micropipette.

It was more difficult to find what we considered to be transplanted RPE in the subretinal space at longer times after transplantation. In only approximately 40% of the retinas was anything suspected of being a transplant found at 8 months or longer after transplantation. These possible transplants were seldom as numerous as they were at earlier times after surgery and contained less pigment than normal. In some cases they appeared to have degenerated in comparison with the host RPE. We found no evidence of unusual cells in the subretinal space or choroid suggestive of inflammation.

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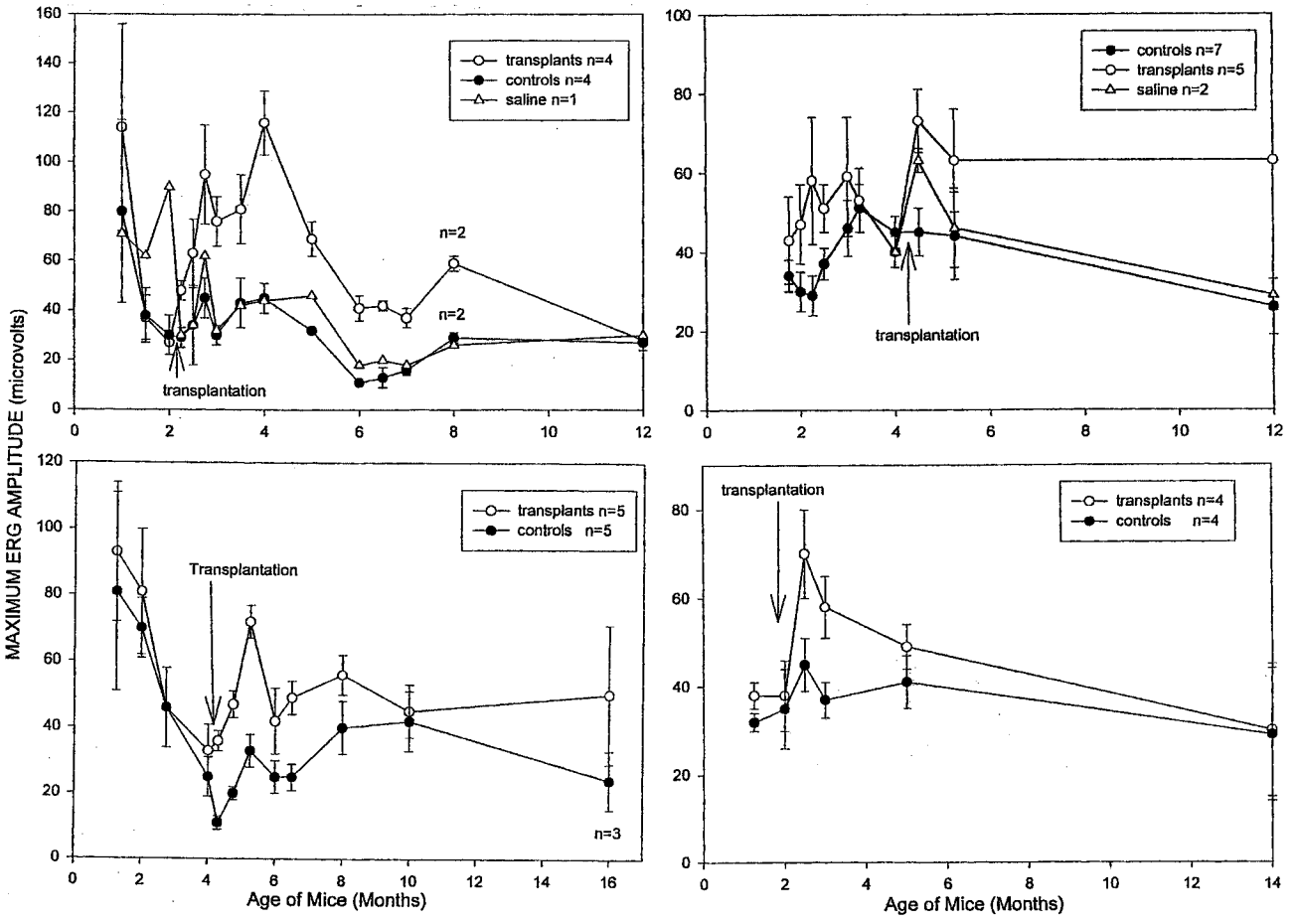

FIGURE 1. Average ERG amplitudes from each eye of four litters of Rpe $65^{-/-}$ mice, before and after receiving RPE transplants in one eye. The other eye received control treatment or was untouched. In three mice, the control was a subretinal injection of saline. *Arrow*: age at which each litter received the transplants. The number of mice in each litter is shown at *top right*. In some cases, mice from a litter were killed before others, reducing the number of responses averaged. *Vertical bars*: SEM.

DISCUSSION

The results show that RPE transplantation produced a transient increase of ERG function in the Rpe$65^{-/-}$ mutant retina. Sham surgery involving injections of saline rather than normal RPE cells was ineffective. The greater amplitude of the ERG in the eye receiving the transplants could be due to an increased availability of the 11-*cis* isomer of retinal to the photoreceptors

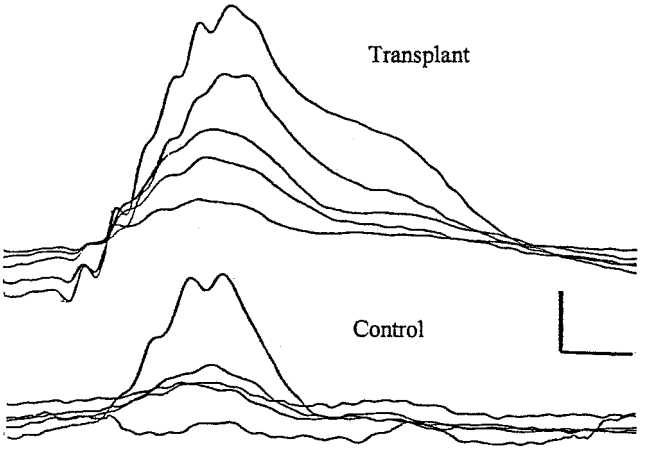

FIGURE 2. ERGs from an eye that had received an RPE transplant 3 weeks before (*top*) compared with the control eye, which was not touched (*bottom*). The calibration on the *right* indicates 10 μV vertically and 35 msec horizontally. Responses to stimuli of different flash intensities over a range of 2 log units are superimposed.

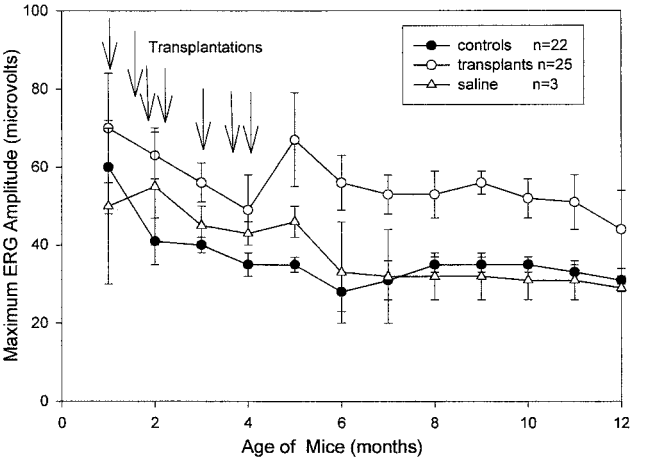

FIGURE 3. Relationship between the average ERG responses in control and test eyes and the age of the mice in all seven litters. Three control subjects received an injection of saline. The other control eyes were untouched. *Arrows*: age of the mice when mice received transplants. *Vertical bars*: SEM.

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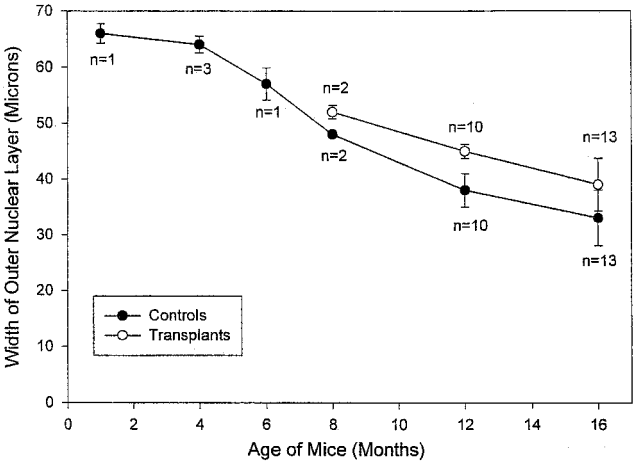

FIGURE 4. Relationship between the maximum width of the outer nuclear layer in the retinal area receiving RPE transplants and the age of control and test Rpe65$^{-/-}$ mice. The number of mice studied at each time point is shown above each data point. *Vertical lines*: SEM.

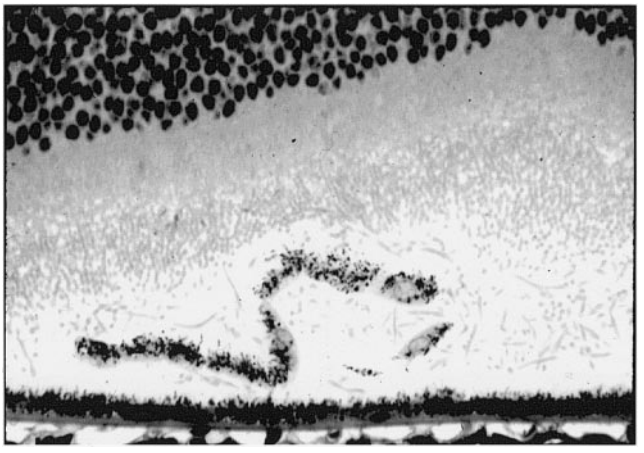

FIGURE 6. Light micrograph of suspected RPE transplants in the subretinal space at 10 days after surgery.

delivered from stores in the transplant and/or synthesized by the transplant. It is also possible that these RPE cells exert a rescue effect on the slowly degenerating photoreceptors unrelated to vitamin A metabolism. The increase in amplitude of the ERG never reached that of a normal mouse, which at maximum amplitude ranges from 500 to 1000 μV. The maximum amplitudes attained from transplantation are approximately 10% to 20% of this value. We presume this was because we could only influence a small area of the retina and perhaps inefficiently.

The reason for this loss of a therapeutic effect with time may be due to the degeneration of the transplanted RPE. This hypothesis is based on the observation that putative RPE transplants were easier to find and healthier in appearance at early than at later times after surgery. This interpretation must be taken with a caveat because our experimental protocol did not include unequivocal markers for the transplants. There was no evidence of inflammation in these retinas, which suggests that transplants were being rejected, but we could have missed this by not sampling shortly after the rescue effect began to decline. In addition, we did not examine these retinas with methods to detect specific T-cells that could unequivocally demonstrate a rejection response.

The long-term survival of RPE transplants is still poorly understood. Some RPE xenografts can survive for at least 6 months subretinally in monkeys,[17] and allografts can survive in rats for at least 1 year[18–20] and in humans for several years.[21] In general, patch transplants of RPE appear to survive longer than dissociated cell transplants.[21] There is evidence that patch transplants are less prone than dissociated cells to rejection.[22] In general, however, only a fraction of such allografts or xenografts seem to survive indefinitely,[23,24] and this has been thought to reflect host–graft rejection. Others find that RPE allografts in rabbits will degenerate even in the presence of immunosuppression[25,26] or before there is a chance for rejection[27] to occur. Transplants may also degenerate because of their abnormal position on top of the host RPE layer, perhaps because it is not an ideal substrate.[28] In the RCS rat, RPE transplants adjacent to the host RPE layer rescue photoreceptors from degenerating for at least 1 year,[18–20] although there is evidence of a diminishing effect due to a noncellular form of rejection.[29]There may be factors unrelated to host–graft rejection contributing to the long-term survival of RPE transplants, which would be valuable to understand if this approach is to advance.

A similar RPE65 gene defect occurs in Briard dogs. This degeneration can be rescued by introducing the normal Rpe65 gene into the RPE by an adenoassociated viral vector. This also leads to an increase in ERG amplitude and in addition to behavioral evidence of restored vision.[16] There is also evidence of rescue from the oral administration of 9-*cis* retinal.[15] It will be valuable to determine the effects of such therapeutic strategies over longer periods of time.

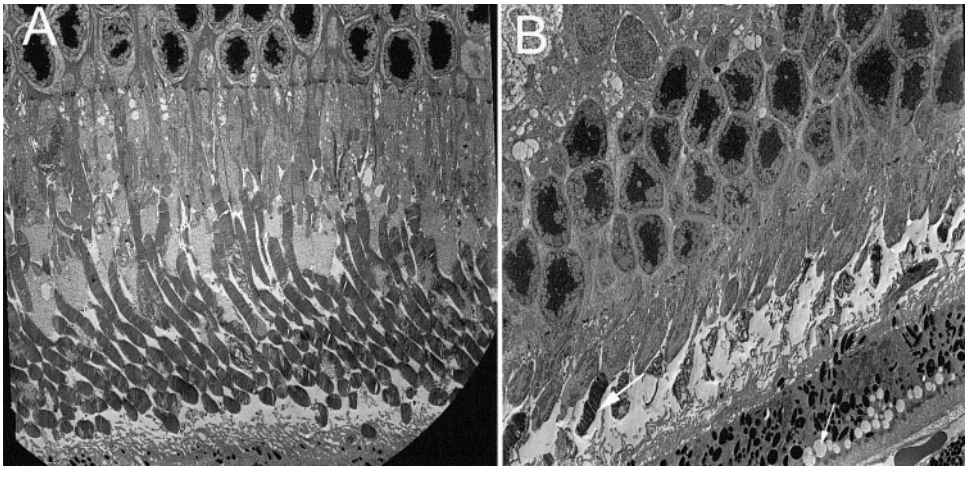

FIGURE 5. Electron micrographs of the photoreceptors of Rpe65$^{-/-}$ mice at 6 months (**A**) and 1 year (**B**) of age. (**B**, *large arrow*) A relatively intact outer segment. (**B**, *small arrow*) Prominent vacuoles in the RPE.

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The photoreceptor degeneration in the original Rpe65$^{-/-}$ strain was found to be slow. The width of the outer nuclear layer was reduced to approximately eight to nine layers of nuclei at 7 weeks and to seven layers of nuclei at 7 months of age.[11] There was no evidence that the amplitude of the ERG, which was thought to be derived from cones, was decreasing. Only rod function was considered defective. Recently, evidence contradicting this interpretation has been published. Breeding experiments that produced a strain of Rpe65$^{-/-}$ mice unable to generate cone responses continued to generate responses leading to the conclusion that the ERG being detected in the Rpe65$^{-/-}$ mice was rod and not cone derived.[30] Ekesten et al.[31] has reported that there is a UV cone defect in Rpe65$^{-/-}$ mice, supporting the hypothesis that cones are affected by the genetic defect. Our results reveal a slow decline of ERG amplitude with time, but the rate of decline diminishes at about 6 months of age, although anatomic degeneration seems to progress. Progression of the degeneration is obviously slow in this retinal degeneration. This implies that a successful therapy should always be able to improve function in this disease. In this regard, our rescue results were similar whether transplantation occurred when the mice were 1 or 4 months of age. Rescue may be possible at any age in this model of retinal degeneration.

It would be valuable to know whether the surviving ERG is exclusively rod generated as indicated by experiments of Seeliger et al.[30] In the human form of this degeneration[8] and in the Briard dog model,[32] both rod and cone systems are affected, but cone ERGs survive longer than rod ERGs, typical of many forms of retinitis pigmentosa. A survival of rod function in the presence of a progressive loss of photoreceptors and absent cone function would make this an unusual model of a retinal degeneration and should prompt a more careful evaluation of ERG components in human forms of this disease.

RPE transplantation can restore function to the photoreceptors in a mouse model of LCA but the therapeutic effect is transient. A positive effect provides useful feedback in future attempts to improve this form of therapy.

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