In Vitro and In Vivo Characterization of Pigment Epithelial Cells Differentiated from Primate Embryonic Stem Cells

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Purpose. To determine whether primate embryonic stem (ES) cell-derived pigment epithelial cells (ESPEs) have the properties and functions of retinal pigment epithelial (RPE) cells in vitro and in vivo.

Methods. Cynomolgus monkey ES cells were induced to differentiate into pigment epithelial cells by coculturing them with PA6 stromal cells in a differentiating medium. The expanded, single-layer ESPEs were examined by light and electron microscopy. The expression of standard RPE markers by the ESPEs was determined by RT-PCR, Western blot, and immunocytochemical analyses. The ESPEs were transplanted into the subretinal space of 4-week-old Royal College of Surgeons (RCS) rats, and the eyes were analyzed immunohistochemically at 8 weeks after grafting. The effect of the ESPE graft on the visual function of RCS rats was estimated by optokinetic reflex.

Results. The expanded ESPEs were hexagonal and contained significant amounts of pigment. The ESPEs expressed typical RPE markers: ZO-1, RPE65, CRALBP, and Mertk. They had extensive microvilli and were able to phagocytose latex beads. When transplanted into the subretinal space of RCS rats, the grafted ESPEs enhanced the survival of the host photoreceptors. The effects of the transplanted ESPEs were confirmed by histologic analyses and behavioral tests.

Conclusions. The ESPEs had morphologic and physiological properties of normal RPE cells, and these findings suggest that these cells may provide an unlimited source of primate cells to be used for the study of pathogenesis, drug development, and cell-replacement therapy in eyes with retinal degenerative diseases due to primary RPE dysfunction. (*Invest Ophthalmol Vis Sci.* 2004;45:1020–1025) DOI:10.1167/iovs.03-1034

The retinal pigment epithelium (RPE) forms a single layer of highly specialized pigmented cells located distal to the photoreceptor cells that performs critical functions in the maintenance of the physiology of the photoreceptors. These functions include the absorption of stray light by its melanin granules, formation of the blood–retinal barrier, regeneration of visual pigments, and phagocytosis of shed outer segments of photoreceptors.[1] RPE cell dysfunction caused by environmental and/or genetic mutations can lead to ocular diseases such as age-related macular degeneration and some forms of retinitis pigmentosa. At present, transplantation of the fetal RPE cells is performed in eyes with ocular diseases with primary RPE dysfunction[2]; however, obtaining a sufficient number of suitable donor cells remains a problem.

Embryonic stem (ES) cells retain significant developmental potential and replicative capability and are expected to alleviate the problem of the shortage of donor cells for cell-replacement therapy. The isolation and use of human ES cells[3,4] has drawn much attention because of their potential clinical applications in patients with degenerative diseases. However, the use of human ES cells for cell-replacement therapy is questionable at the moment because their differentiation is poorly controlled. Compared with the extensive potential demonstrated by mouse ES cells,[5,6] there is no reported case showing that primate ES cells can be successfully applied to animal disease models. As the characteristics of rodent ES cells differ considerably from those of primate ES cells,[3,4,7,8] it is necessary to develop methods to induce primate ES cells to differentiate into a homogeneous population of functional cells that can be used for cell-replacement therapy.

The purpose of the study was to determine whether primate embryonic stem-cell–derived pigment epithelial cells (ESPEs) develop the well-known characteristics of RPE cells and have functional properties that would be of value in treating diseases when transplanted in an animal model of RPE dysfunction.

Materials and Methods

Cell Culture

A cynomolgus monkey ES cell line was obtained from Asahi Techno Glass Co. (Tokyo, Japan), and the undifferentiated ES cells were maintained as described.[8] The methods used to induce undifferentiated ES cells to differentiate into ESPEs have been described in detail.[9–12] In brief, undifferentiated primate ES cells were plated on PA6 stromal cells and cultured in the differentiation medium for 3 weeks. For the expansion of ESPEs, the dishes were coated with thin synthetic matrix

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(dilution of 1:20; Matrigel) according to the manufacturer's protocol (BD Biosciences, Bedford, MA). The ESPEs were selectively removed with disposable scalpels and plated on the matrix-coated dishes in DMEM supplemented with 10% FBS and 20 ng/mL bFGF.

RT-PCR Analysis

Total RNA was isolated (RNeasy Protect Mini Kit with RNase-Free DNase Set; Qiagen, Chatsworth, CA) and first-strand cDNA was synthesized (First-Strand cDNA Synthesis Kit; Amersham Biosciences, Piscataway, NJ) according to the manufacturer's protocol. The PCR reaction was performed with the following primers: for RPE65, 5′-TGGAGTCTTTGGGGAGCCAA-3′ and 5′-CTCACCACCACACTCAGAAC-3′; for cellular retinaldehyde-binding protein (CRALBP), 5′-GTGGACATGCTCCAGGATTC-3′ and 5′-CCAAAGAGCTGCTCAGCAAC-3′; for Mertk, 5′-GGGAGATCGAGGAGTTTCTC-3′ and 5′-CGGCCTTGGCGGTAATAATC-3′; for β-actin, 5′-CTTCAACACCCCAGCCATGT-3′ and 5′-ACTCCTGCTTGCTGATCCAC-3′.

Western Blot Analysis

Western Blot Analysis was performed as described.[11] Rabbit polyclonal anti-CRALBP antibody (1:40,000, kindly provided by John C. Saari, University of Washington, Seattle, WA) was used as the primary antibody.

Animals

All animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University. Pink-eyed dystrophic Royal College of Surgeons (RCS) rats and congenic nondystrophic rats were obtained from CLEA Japan (Tokyo, Japan).

Transplantation Procedures

Patches of ESPEs were collected by carefully cutting the peripheral margins with disposable scalpels. The patches of ESPEs were gently dissociated with the Papain Dissociation System (Worthington Biochemical, Lakewood, NJ) according to the manufacturer's protocol. Dissociated ESPEs were incubated in the CM-DiI (chloromethylbenzamido derivatives of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; Molecular Probes, Eugene, OR) solution at a concentration of 5 μg/mL for 20 minutes at 37°C. Labeled ESPEs were then washed three times with PBS. The viability of the ESPEs after these procedures was more than 95%, as assessed by trypan blue exclusion. The cells were centrifuged and then concentrated to approximately 10,000 cells/μL in PBS.

The surgical and grafting procedures have been described in detail.[13,14] ESPE cells, suspended in 3 μL of PBS, were injected transsclerally into the dorsotemporal subretinal space of anesthetized 4-week-old RCS rats. All transplantations were made into the left eye. Sham-treated RCS rats received the same amount of carrier medium. A total of 41 RCS rats received ESPE grafts, and 21 had sham injection. Transplantation into the subretinal space was confirmed by direct observation of the rat fundus with a contact lens (Kyocon, Kyoto, Japan), and those that had successful transplantation were selected for histologic analyses and behavioral tests. All the animals were maintained on oral cyclosporine (200 mg/L in drinking water; Calbiochem, Darmstadt, Germany) from 2 days before transplantation until they were killed. The blood cyclosporine levels in these animals were measured by SRL Inc. (Tokyo, Japan).

Immunostaining

Standard immunocytochemical techniques were used for the in vitro studies.[15] The working dilution of the rabbit polyclonal anti-ZO-1 antibody (Zymed, South San Francisco, CA) was 1:50. Eyes ($n = 4$ for each group) were harvested 8 weeks after transplantation at age 12 weeks and fixed in 4% paraformaldehyde. Sixteen-micrometer sections were cut with a cryostat, stained, and processed for light or transmission electron microscopy as described.[16]

The working dilution of the mouse monoclonal anti-rhodopsin antibody (Sigma-Aldrich, St. Louis, MO) was 1:2000. The nuclei were stained with Cytox blue (1:500 in distilled water; Molecular Probes) and the specimens were observed and photographed with a laser-scanning confocal microscopy (TCS SP2; Leica, Heidelberg, Germany). The maximum thickness of the outer nuclear layer (ONL) in the dorsotemporal and ventronasal retina ($n = 4$ animals for each group) was measured, and the differences were analyzed with the Mann-Whitney test.

Transmission Electron Microscopy and Phagocytosis of Latex Beads

ESPEs grown on 60 mm synthetic-matrix–coated dishes (Matrigel; BD Biosciences) were processed for transmission electron microscopy as described.[16] To examine phagocytotic ability,[17] the ESPEs were incubated with 1-μm latex beads (Sigma-Aldrich) at a concentration of 1.0×10^9 beads/mL for 6 hours at 37°C. The ESPEs were washed five times with PBS and then processed for transmission electron microscopy.

Behavioral Assessment

For behavioral assessment, a head-tracking apparatus (Hayashi Seisakusho, Kyoto, Japan) that consisted of a circular drum rotating around a stationary holding chamber containing the animal was used.[13] The speed of rotation of the drum with vertical black-and-white stripes (10° each) was set at 2, 4, and 8 rpm. Animals ($n = 4$ animals for each group) were tested at 8 weeks after transplantation at 12 weeks of age before they were killed. A video camera mounted above the apparatus recorded the head movements. The total amount of head-tracking time was determined at speeds of 2, 4, and 8 rpm during a 4-minute test period for each speed. A single operator, masked to the type of animals being tested, conducted all assessments, and the code was broken after the completion of all data acquisition. Behavioral data were analyzed with the Mann-Whitney test.

Results

In Vitro Characterization of ESPEs Differentiated from Primate ES Cells

We have reported an efficient method to induce differentiation of cynomolgus monkey ES cells into pigment epithelial cells in vitro.[9] To determine whether these primate ESPEs possessed the characteristics of RPE cells, clusters of ESPEs were selected and expanded into a uniform single cell layer on matrix-coated dishes (Fig. 1A). These ESPEs reproducibly exhibited a hexagonal shape, and each cell contained a significant amount of melanin pigments (Fig. 1B). Transmission electron microscopy of ESPEs showed that these cells had the typical structures of the RPE such as extensive apical microvilli and numerous pigment granules (Fig. 2A). These findings indicated that the ESPEs have the morphologic appearance of RPE cells.

The blood–retinal barrier strictly regulates the microenvironment of the photoreceptors, and the RPE cells contribute to this barrier function by forming tight junctions between adjacent cells. Immunocytochemical analysis showed strong staining of the cell junctions between ESPEs by ZO-1, a tight junction protein, indicating that ESPEs form tight junctions in vitro (Figs. 1C–E).

We next examined the expression of specific molecules closely related to the cellular function of normal RPE cells (i.e., RPE65 and CRALBP, both of which are involved in regeneration of visual pigment and are strongly expressed in normal RPE cells).[18,19] Mertk, a tyrosine kinase receptor gene, is essential for the phagocytosis of photoreceptor outer segments

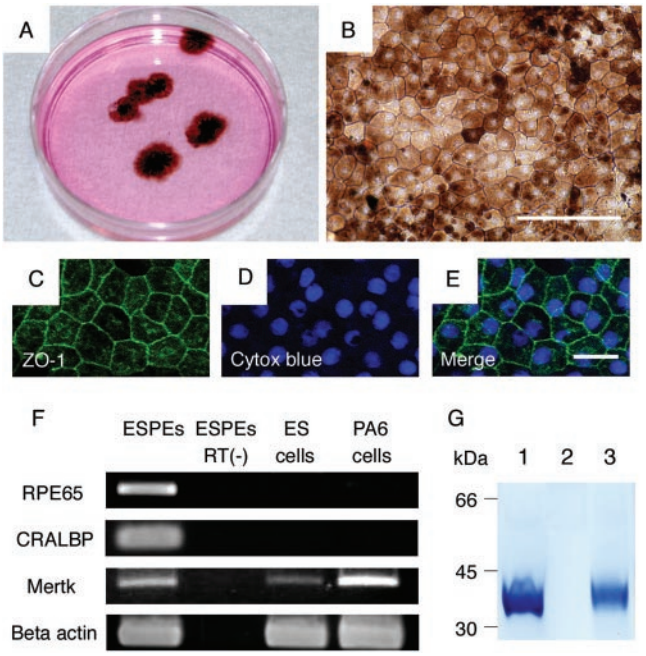


FIGURE 1. Characterization of pigment epithelial cells differentiated from primate ES cells. (**A**) Clusters of ESPEs were selected and expanded as patches of a uniform single cell layer on a 60-mm synthetic-matrix–coated dish. (**B**) The expanded ESPEs had a hexagonal shape with significant amounts of pigment. (**C–E**) Immunocytochemical staining showed positivity to ZO-1, a tight junction protein, in the ESPEs. ZO-1 (**C**, *green*), nuclei in cells stained with Cytox blue (**D**, *blue*), and combined (**E**). (**F**) RT-PCR analysis of RPE gene expression by differentiated ESPEs, differentiated ESPEs with reverse transcriptase omitted, undifferentiated ES cells, and PA6 stromal cells. (**G**) Western blot analysis of CRALBP expression in ESPEs. Cell lysates from RPE (*lane 1*), undifferentiated ES cells (*lane 2*), and differentiated ESPEs (*lane 3*) were probed with the anti-CRALBP antibody. Scale bar: (**B**) 100 μm; (**C–E**) 20 μm.

by RPE cells[20,21] and is expressed not only in RPE but also in undifferentiated ES cells and in various hematopoietic cell lines.[22] RPE65, CRALBP, and Mertk are essential for normal visual functions, because a mutation in any of these three genes in humans causes visual disturbances.[23–26] RT-PCR detected the expression of the mRNA of RPE65, CRALBP, and Mertk in the ESPEs. In addition, Western blot analysis confirmed the expression of the CRALBP protein in the ESPEs, which yielded a single band of the appropriate size (Fig. 1G).

To function and be viable, photoreceptor cells require a continuous phagocytosis of their shed outer segments by adjacent RPE cells.[1] There are two separate mechanisms for phagocytosis in RPE cells in vitro: a nonspecific process (as seen with the uptake of latex beads) and a specific uptake of shed outer segment fragments involving a receptor-mediated event. To examine whether ESPEs had phagocytic capability, they were incubated with 1-μm fluorescent latex beads.[17] When observed by a fluorescence light microscope, the abundant melanin granules in the ESPE cytoplasm obscured the bead-specific fluorescence. However, transmission electron microscopy clearly showed that the ESPEs had ingested the latex beads (Fig. 2B).

Transplantation into a Rat Model with RPE Dysfunction

RCS rats show a progressive photoreceptor loss, which is mostly marked during the first 3 months after birth.[27] Retinal degeneration in the RCS rat is primarily due to the failure of the RPE cells to phagocytose shed outer segments,[28] which is the result of a mutation of the receptor tyrosine kinase gene (*Mertk*).[29] Subretinal transplantation of fetal RPE cells into the dystrophic RCS rat at an early age resulted in structural and functional preservation of photoreceptors.[30,31]

We used this animal model to explore the ability of ESPEs to rescue the function in host animals. The host animals were given cyclosporine to prevent xenograft rejection of the monkey ESPEs. At the termination of the experiments, the mean blood cyclosporine level in these animals was 244 ± 73.0 ng/mL, and there was no histologic evidence of any inflammatory immune reaction at the site of cell injection.

When the animals were 12 weeks old (8 weeks after transplantation), the heavy pigmentation of the ESPEs made it easy to identify them in the pink-eyed host RPE cells phagocytosing pigment debris of donor cells (Fig. 3A). Prelabeling the ESPEs with CM-DiI also confirmed that these heavily pigmented cells in the host subretinal space were derived from the donor cells (Figs. 3A–C).

We also measured the thickness of the ONL to determine whether the photoreceptor cells were rescued by the ESPE transplantation. The normal thickness of photoreceptors in the ONL of the congenic nondystrophic rat retina was 11 to 12 cells thick (Fig. 3D), and in the nonsurgical RCS rat retina, the ONL was reduced to an occasional cell lying at the outer border of the inner nuclear layer (INL). In the sham-surgery RCS rat retina, the thickness of the ONL was two to three cells thick in the small area immediately surrounding the injection site (Fig. 3E). When ESPEs were transplanted into the RCS rat retina, however, the ONL directly above the pigmented ESPEs consisted of photoreceptor cell nuclei up to eight cells thick (Figs. 3B, 3F).

The maximum ONL thickness was significantly greater in the ESPE-grafted RCS rat group than in the sham-treated RCS rat group or in the untreated RCS rat group (Fig. 3G; Mann-Whitney analysis, $P < 0.05$). In every ESPE-grafted eye, the maximum ONL thickness of the dorsotemporal retina (ESPE-grafted quadrant) was greater than that of the ventronasal retina of the same eye (Fig. 3G). In contrast, in the nonsurgical eyes, there was no significant difference in the maximum ONL thickness between the dorsotemporal and the ventronasal retina (Fig. 3G).

Immunohistochemical analysis showed that the preserved photoreceptors expressed rhodopsin, visual pigment used by the rod photoreceptor cells to perform phototransduction (Fig. 3F). Electron microscopy of the grafted ESPEs revealed the presence of lamellar structures within the pigmented ESPEs (Fig. 4). These results indicate that the ESPEs developed a

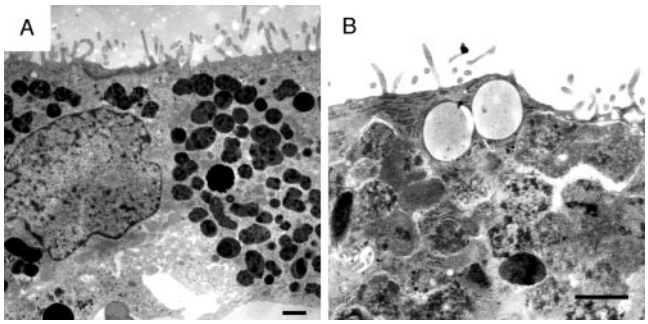
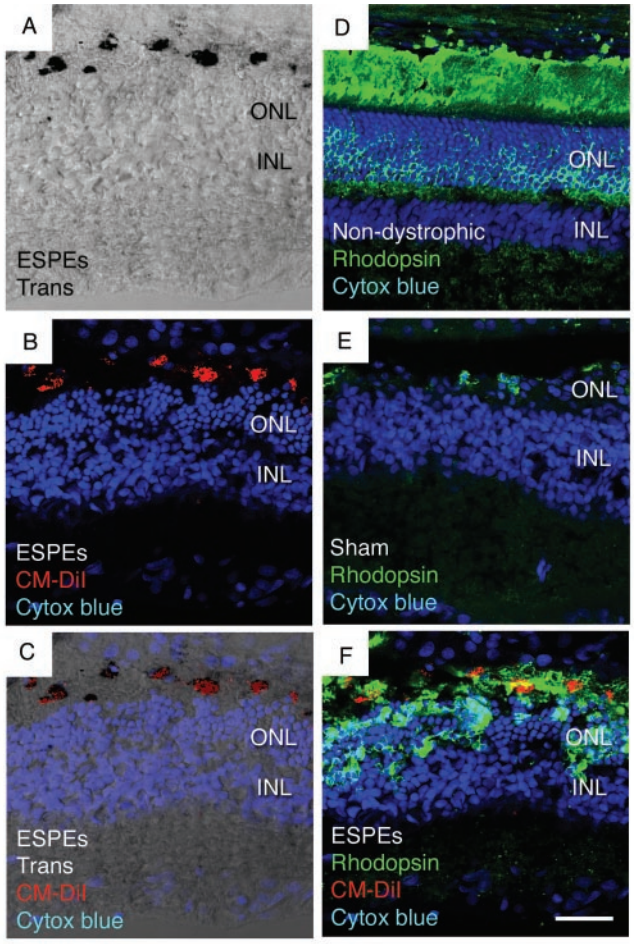


FIGURE 2. Transmission electron microscopy of pigment epithelial cells differentiated from primate ES cells. (**A**) ESPEs had the typical structures of the RPE, such as extensive apical microvilli and numerous pigment granules. (**B**) ESPEs had the ability to incorporate 1-μm latex beads. Scale bars, 1 μm.



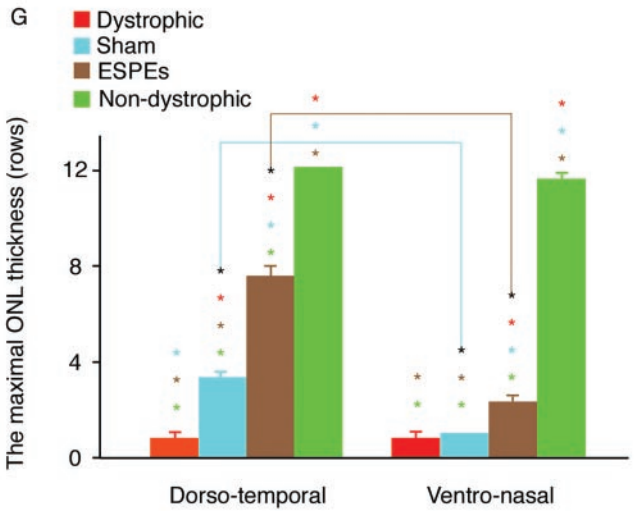


FIGURE 3. Immunohistochemical analyses after ESPE transplantation into RCS rats. (**A–C**) ESPE-grafted RCS rat retina at the age of 12 weeks (8 weeks after transplantation). (**D–F**) The expression of rhodopsin (*green*) in the photoreceptors at the age of 12 weeks. Congenic nondystrophic rat retina (**D**). Sham-surgery RCS rat retina (**E**). ESPE-grafted RCS rat retina (**F**). Nuclei in cells stained with Cytox blue (**B–F**, *blue*). The grafted ESPEs prelabeled with CM-DiI (**B**, **C**, **F**, *red*). (**G**) Histologic analysis at the age of 12 weeks. The maximum ONL thickness of the dorsotemporal retina (grafted quadrant) and that of the ventronasal retina (four animals in each group). Error bars, SD. *Colored asterisks*: statistical difference ($P < 0.05$, Mann-Whitney analysis) in the maximum ONL thickness compared with the nonsurgical RCS rat group (*red*), the sham-surgery RCS rat group (*blue*), the ESPE-grafted RCS rat group (*brown*), and the nondystrophic rat group (*green*). *Black asterisks*: significant difference ($P < 0.05$, Mann-Whitney analysis) in the maximum ONL thickness between the dorsotemporal and ventronasal retina. Scale bar, 80 μm.

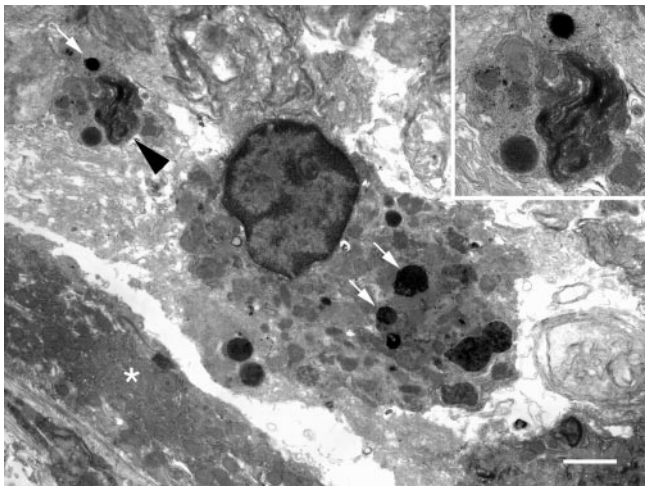


FIGURE 4. Electron microscopy of the grafted ESPEs. The presence of lamellar structures within the grafted ESPEs (*arrowhead*). *Inset*: higher magnification. *Arrows*: pigment granules in the ESPE cytoplasm. *Asterisk*: host RPE. Scale bar, 2 μm.

capability to promote the survival of photoreceptor cells in an animal model of RPE dysfunction.

Behavioral Assessment of Transplant Recipients

To determine whether the transplantation preserved visual function, each of the transplant-recipient animals was placed in a clear glass container surrounded by a motor-driven rotating drum with vertical black-and-white stripes (Fig. 5A). The visual function of the animals was estimated by measuring the head-tracking time in response to the rotating stripes, which is closely associated with the optokinetic reflex.[13] At the age of 12 weeks (8 weeks after the operation), the head-tracking time of the ESPE-grafted RCS rat group was significantly longer than that of the untreated RCS rat group (Fig. 5B; Mann-Whitney analysis, $P < 0.05$), but was not significantly different from that of the nondystrophic rat group. In contrast, the head-tracking time of the sham-treated RCS rat group was significantly shorter than that of the nondystrophic rat group (Fig. 5B, Mann-Whitney analysis, $P < 0.05$), but not significantly different from that of the untreated RCS rat group. These results indicate that ESPEs could preserve a significant level of visual function when transplanted into an animal model of RPE dysfunction.

DISCUSSION

One of the advantages of using human ES cells for the treatment of degenerative diseases is that these cells have the capacity to provide an unlimited source of specific cell types. However, methods for purifying large numbers of lineage-specific cells should be developed for clinical application. In this study, we demonstrated that pigment epithelial cells can be generated, enriched, and expanded from primate ES cells. These ES-converted pigment epithelial cells showed development of several of the characteristics of RPE cells and were able

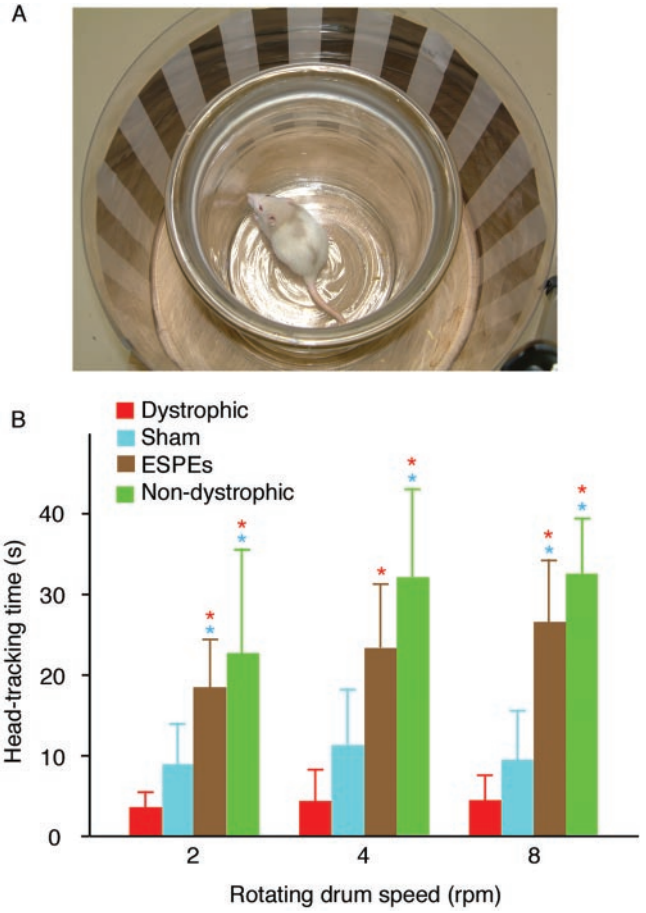


FIGURE 5. Behavioral assessment after ESPE transplantation into RCS rats. (**A**) Photograph of head-tracking apparatus. (**B**) The total amount of head-tracking time to rotating stripes at the speeds of 2, 4, and 8 rpm during the 4-minute test period for each speed (four animals in each group). Error bars, SD. *Asterisks*: significant difference ($P < 0.05$, Mann-Whitney analysis) in the head-tracking time compared with the nonsurgical RCS rat group (*red*) and the sham-surgery RCS rat group (*blue*).

to attenuate the loss of photoreceptors when transplanted subretinally into RCS rats.

The grafted ESPEs probably preserved the photoreceptors in the RCS rat retina, either by phagocytosing the host's outer segments[1] or by secreting soluble growth factors.[32] Although ESPEs are able to phagocytose latex beads (Fig. 2B), we have not measured their ability to phagocytose photoreceptor outer segments in vitro. However, phagosome-like bodies were seen in the grafted ESPEs by electron microscopy (Fig. 4), suggesting that grafted ESPEs had the ability to ingest host shed outer segments. The results of transplantation may have been even better if we could have transplanted an organized patch of ESPEs instead of dissociated cells, because, in such a patch, cellular polarity and tight junctions seem more likely to develop.

An earlier study reported the retinal transplantation of neural precursors that had been differentiated from mouse ES cells.[33] The transplanted cells probably slowed the photoreceptor degeneration in RCS rats by secreting some growth factors, because grafted neural precursors can neither phagocytose host shed outer segments nor differentiate into photoreceptor cells. In contrast, our results showed that ESPEs from primate ES cells can differentiate and develop characteristic properties of RPE cells, which would be necessary for the treatment of primary RPE dysfunction and for the long-term preservation of visual function after retinal transplantation.

If undifferentiated cells are contaminated, transplantation of ES cell-derived cells might involve the risk of tumor formation in the host animal. However, no tumors were observed in the animals that received ESPE grafts in our study. One reason for this may be that we selectively expanded the ESPEs as patches of cells on the matrix-coated dishes and generated relatively pure populations of donor pigment epithelial cells. These procedures may have kept unwanted cell populations from contaminating the donor cells.

Because both undifferentiated primate ES cells and ESPEs can be expanded in vitro, it is possible to generate an unlimited number of ESPEs for retinal transplantation. Considering the close phylogenetic relationship between humans and cynomolgus monkeys, we can also expect that the methods of differentiation used to generate ESPEs can be applied to human ES cells. Human retinal diseases for which ESPE transplantation may be used include age-related macular degeneration and hereditary retinal degeneration due to primary RPE dysfunction, such as some forms of retinitis pigmentosa.

One substantial problem to be solved is the control of immunologic rejection after the transplantation of allograft tissue. It is therefore important to determine in future studies how much immunosuppression is necessary after ESPE transplantation into the subretinal space, which is sometimes regarded as an immunologically privileged site for retinal allografts.[34] The transplantation of monkey ESPEs into the monkey subretinal space would provide a more accurate model for the allograft transplantation of human ESPEs into other humans.

Our results indicate that the expected morphologic, biochemical, and functional characteristics of RPE cells developed in the expanded ESPEs in vitro. After transplantation of the ESPEs into the subretinal space of an animal model of RPE dysfunction, the grafted ESPEs enhanced the survival of host photoreceptors. These effects were demonstrated both by histologic analyses and behavioral tests. To the best of our knowledge, this is the first study to show detailed functioning of specific cells differentiated from primate ES cells both in vitro and in vivo. In addition, this is also the first study to demonstrate the successful therapeutic application of primate ES cells in an animal disease model. As human ES cells significantly differ from mouse ES cells but closely resemble nonhuman primate ES cells, the latter would be more suitable for preclinical research aimed at cell-replacement therapies. Before human ESPEs are used in clinical trials, however, long-term studies of retinal transplantation in nonhuman primate hosts are necessary to confirm the cells' safety and efficacy.

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