Adequate Time Window and Environmental Factors Supporting Retinal Graft Cell Survival in *rd* Mice

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Postnatal photoreceptor cells can be integrated into the wild-type adult retina in mice, and retinal transplantation is now one therapeutic option for retinal degenerative diseases when photoreceptor degeneration is the primary cause of the disease. The aim of this study was to specify the optimal time window during the course of retinal degeneration and to modulate the host and/or graft environment for a successful transplantation. We first studied the background features of the mice with phosphodiesterase 6b (PDE6b) gene mutation (*rd*; C3H/Hej) and found that the infiltration of microglia and glial fibrillary acidic protein (GFAP) expression once increased at the peak of rod death (~2–3 weeks of age) but then reduced for a following period until gliosis began to take place with enhanced GFAP expression (~8 weeks of age). The postnatal retinal cells (p4–p7) were successfully transplanted during this period with neurite extension into the host retina. In later transplantations (6 or 8 weeks of age), graft cells survived better in the presence of chondroitinase ABC (ChABC), which digests chondroitin sulfate proteoglycan (CSPG), an essential component of gliosis. In contrast, in earlier transplantations (4 weeks of age), graft cells survived better in the presence of valproic acid (VPA), a neural differentiating reagent, or glatiramer acetate, an immune modulator. These suggest that, immediately after the outer nuclear layer (ONL) degeneration, an inflammatory reaction may be easily induced but the host neurons may be more able to accept donor cells in the presence of neural differentiating factor. These results will help optimize transplantation conditions when we consider clinical application.

Key words: Photoreceptors; Transplantation; Gliosis; Microglia; Glatiramer acetate; Valproic acid

INTRODUCTION

It was previously shown that photoreceptors of a specific development stage can be incorporated into the adult retina of wild-type mice and acquire mature photoreceptor properties (16). This suggested that photoreceptor cell transplantation is a practical therapeutic option and a promising approach for retinal degenerative diseases when photoreceptor degeneration is the primary cause of the disease. When we consider a clinical application of transplantation therapy, the first step trial would be the transplantation of graft cells into the subretinal space of degenerated retina with minimum photoreceptor function, possibly with no structural outer nuclear layer (ONL). The optimized conditioning of the host retina for the integration of transplanted photoreceptors with no ONL, however, has not been studied in detail. For this purpose, we used the murine model with rapid retinal degeneration with phosphodiesterase 6b (PDE6b) gene mutation (*rd*; C3H/Hej) to study the graft cell survival in the course

of degeneration, especially focusing on the timing immediately after rod death (4). In *rd* mice, although abnormality of the remaining inner retina was also reported from the early degeneration period (8,29), some transplantation studies reported restoration or maintained visual function when retina sheets had been transplanted in young *rd* mice (3,26). Progressive retinal degeneration is often accompanied by an inflammatory reaction represented by infiltration of microglia that can prevent graft integration (28,33), resulting in difficult incorporation of transplanted cells into host retina. In the later stage of degeneration, a gliotic change reportedly takes place that inhibits the incorporation of transplanted cells (8). Because successful transplantation in wild-type retina was achieved in the presence of no specific inflammatory or gliotic change other than transplantation injury (32), we propose that transplantation should be performed under conditions of least microglia invasion and less gliotic reaction. To specify the optimal time window for transplantation, we investigated the microglia behavior and

gliotic changes associated with the course of retinal degeneration in *rd* mice. From these observations, we investigated how transplanted cells could be incorporated into the host retina depending on the timing of transplantation. Additionally, we assessed the effects of glatiramer acetate, an immune-modulating and neuroprotective factor, valproic acid (VPA), a neural differentiating factor, and chondroitinase ABC (ChABC), a gliosis modulating factor, on graft cell integration. The rationale to test each of these factors is summarized below.

Glatiramer acetate is a synthetic random amino acid polymer used as a first-line treatment agent for relapsing–remitting multiple sclerosis. The modulation of antigen-presenting cells (APCs) such as monocytes and dendritic cells, CD4⁺ T (h) cells, CD8⁺ T cells, and Foxp3⁺ regulatory T cells by this agent results in anti-inflammatory or neuroprotective properties (7,12,13). Microglia, in addition to their contribution to the immunological or inflammatory environment, may also accelerate the removal of inappropriate synapses (5,31) or inappropriate cells (19) from the neural network. Proper differentiation and accelerated synaptic formation of graft cells are important for their survival and integration. VPA, an antiepileptic drug, was recently reported to promote the neural differentiation of transplanted neural stem cells and to promote the recovery of motor function in spinal cord injury models (1). We additionally reported that proliferating Müller glia after injury more efficiently differentiated into photoreceptors with VPA treatment in mice explants as a regenerative process (24). ChABC was reported to digest chondroitin sulfate proteoglycans (CSPGs), which inhibit neurite extension in newly born and transplanted cells (20). In retinal degeneration, Müller cells gradually migrate to the outer borders of the retina to form a thick seal of cell processes to create a nonpermissive environment for graft cells, and CSPGs presumably take part in this process (11). We previously showed that treatment with ChABC at the time of transplantation enhanced the number of surviving graft cells with extended neurites into the host retina in a chemical retinal degeneration model (30).

MATERIALS AND METHODS

Experimental Animals

All animals were cared for in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and the Guidelines of Riken CDB Animal Experiment Committee. C3H/HeJ Yok Slc strain mice (*rd* mice) were purchased from Nihon SLC (Shizuoka, Japan). C57Bl/6J, Nrl.gfp^{+/+} (green fluorescent protein) transgenic mice were initially provided by Dr. A. Swaroop at the University of Michigan and were maintained in the animal facility at Kobe Riken CDB. The animals were maintained under standard laboratory

conditions (18–23°C, 40–65% humidity, and a 12-h light–dark cycle) with free access to food and water throughout the experimental period.

Background Characteristics of rd Mice

Background characteristics were studied immunohistochemically in the course of retinal degeneration in *rd* mice. The eyes of *rd* mice were fixed in Superfix (Kurabo, Japan) at postnatal weeks 2, 3, 4, 7–8, and 12–14 and were embedded in paraffin for sectioning. The sections were used for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining and immunostaining.

Transplantation Procedure

Graft cells were prepared from p3–p7 Nrl-GFP mice (B16). Retinal cells were treated with Accutase (Sigma, St. Louis, MO) for 15 min at 37°C. After gentle pipetting, the cells were resuspended in Hank's balanced salt solution (HBSS)-glucose (50 mg/L) at a concentration of 2×10^5 cells/ μ L. The cell suspension was injected (3 μ L/eye) under the mouse retina using a FemtoJet Microinjector (Eppendorf, Germany). ChABC at 25 mU/ μ L (Wako, Tokyo, Japan) or VPA (1 mM unless otherwise noted) (Sigma, St. Louis, MO) was added to the cell suspension where indicated. For the immunization of glatiramer acetate, glatiramer acetate/copolymer 1 (COP-1) (10 g/mL; Teva, North Wales, PA) emulsified in Complete Freund's adjuvant (CFA) (Sigma Aldrich) was injected by the subcutaneous (SC) route 1 week before transplantation as described by Gorantla et al. (7).

Indomethacin (10 mg/L) and cyclosporine (100 mg/L) were added to the drinking water of all transplanted mice starting 3 days before the transplantation. The mice were sacrificed 2 weeks after transplantation, and the eyes were fixed for paraffin embedding.

Immunohistochemistry

The eyes were enucleated and placed in fixative (SUPER FIX, Kurabo, Osaka, Japan) at 4°C overnight. After automatic fixation (Excelsior, ThermoFisher Co., UK), the eyes were embedded in paraffin (P3683; Sigma, St. Louis, MO) and the paraffin blocks were sectioned using an autoslide preparation system (AS-200, Kurabo, Osaka, Japan).

For immunohistochemistry, sections were incubated with a blocking solution containing 5% goat serum in PBS for 1 h followed by incubation with the primary antibody [mouse antirhodopsin at 1:1,000, Chemicon, CA; rabbit anti-GFP at 1:1,000, Abcam, UK; rabbit anti-ionized calcium binding adaptor molecule 1 (Iba-1) at 1:500, Nacalai, Japan; mouse anti-Bassoon at 1:500, Abcam, UK; rabbit antirecoverin at 1:1,000, Chemicon, CA] or biotinylated lectin peanut agglutinin (PNA) at 5 μ g/mL (J-OIL MILLS Inc., Tokyo, Japan) in 1% goat serum in

PBS at 4°C overnight. In the next morning, the samples were washed with PBS-0.05% Tween20 (PBS-T) and incubated with the secondary antibody (anti-mouse/rabbit/rat IgG conjugated with cyanine 2 (Cy2)/Cy3/Cy5 at 1:500; Jackson ImmunoResearch, West Grove, PA) and 4',6-diamidino-2-phenylindole (DAPI at 1:1,000; Sigma, St. Louis, MO) in 1% goat serum in PBS for 1 h at room temperature. After washing with PBS-T, the samples were mounted in FluoSave reagent (Calbiochem, San Diego, CA), and the fluorescent images were examined with a confocal microscope (Radiance 2100, BIORAD, Richmond, CA).

For whole-mount staining, retinas were fixed in 4% paraformaldehyde (PFA) for 15 min and incubated with a blocking solution containing 50% goat serum and 20% fetal bovine serum (FBS) in 0.3% Triton-X/PBS for 1 h, and the staining procedure was performed as described above. TUNEL staining was performed using the In situ Cell Death Detection Kit, Fluorescein, according to the manufacturer's protocol (Roche Applied Science).

Cell Counting

We observed each slide at 50- μ m intervals and counted the number of surviving graft cells that were attached to the host retina with neurites extending into or toward the host retina. For convenience, these cells were termed "integrated cells" to indicate morphological integration. These cells were attached to the host retina when the retina was peeled off from the retinal pigment epithelium (RPE), indicating some mechanical interaction between the graft and the host retina. Each number represents the mean of the total count of at least three successful transplantations. For the maturation study with VPA, transplanted eyes were processed for whole-mount staining of the retina on days 4, 5, 6, and 10 after transplantation, and the percentage of rhodopsin-positive cells to GFP-positive photoreceptor precursors was studied in each retina. Unpaired *t* test between with and without factor treatment was performed for statistic evaluations.

RESULTS

Background Feature of *rd* Mice

As reported previously, most rod photoreceptors in *rd* mice were lost in the first 4 weeks after birth (4). We observed many TUNEL-positive cells in the outer nuclear layer (ONL) during the second postnatal week, and these decreased in parallel to ONL degeneration (Fig. 1A). Active microglia also accumulated in the ONL during this period. A transient accumulation of ramified microglia was observed in the inner plexiform layer (IPL) in the third week, but the number of microglia gradually decreased thereafter (Fig. 1A). ZO-1 stains the outer limiting membrane or the junction of photoreceptors and the

Müller feet of the retina. The stain was a clear solid line in the wild-type retina but the line was fragmented in the *rd* mice in the early and later stages of degeneration (3 and 13 weeks, respectively), and the stain was even fainter at 4 and 8 weeks (Fig. 1B). GFAP expression in Müller cells was marked in the second postnatal week, less evident toward the end of rod degeneration, and once again evident in the later weeks (Fig. 1C).

The number of the second neuron rod bipolar cells decreased with time in the retinal sections (Fig. 2A–D). The numbers of cone and the second neuron rod bipolar cells counted on the whole-mount retina also decreased with time after rod loss (Fig. 2E–J). The cone photoreceptors were lost from the central area (Fig. 2G, H, J).

These results indicate that the inflammatory reaction that is reflected by increased GFAP expression and microglia accumulation becomes less evident after rod loss (fourth week) but gliosis and secondary neuronal death occur with time (eighth week). This suggests that the period between the fourth and eighth postnatal weeks may be a good time window for transplantation.

Transplanted Cells in the Degenerating Retina

Cells were well integrated into the wild-type host retina with their neurites properly extended into the outer plexiform layer (OPL) of the host retina (Fig. 3A, B). Integrated transplanted cells often expressed presynaptic marker Bassoon at the terminal bulb (Fig. 3B). In *rd* mice, surviving graft cells often extend neuritis into/onto host retina (Fig. 3C, D). However, the graft cells often failed to extend their Bassoon-positive neurites into the host Bassoon-positive OPL in *rd* retina (Fig. 3C). Although the graft cells remained outside of the remaining ONL, the cells still had a chance to come in touch with the host protein kinase C (PKC)-positive rod bipolar dendrites, the second neuron that receives rod photoreceptor synaptic signals (Fig. 3D). For 3 months after the transplantation, GFP-positive cells had typical dense, small nucleus of photoreceptors, aligned well, positioning close to PKC-positive cells in some sections (Fig. 3E, F). These cells also had inner segment-like accumulated rhodopsin outgrowths (Fig. 3G, H). Graft cells were consistently attached to the host retina when retina was peeled off from the RPE, suggesting some mechanical interaction between these graft cells and the host retina. Therefore, we counted the number of graft cells attached to the host retina with their neurites extending into or toward the host retina as "integrated cells" in subsequent experiments.

Cell Transplantation in the Presence of ChABC

We previously showed that treatment with ChABC at the time of transplantation increases the number of graft cells with neurite projection toward the host retina in a chemical-induced retinal degeneration (30). We observed

Figure 1. Background features of *rd* mice retina during the course of degeneration. (A) TUNEL staining and microglia accumulation at each indicated weeks of age. Top row: Green, TUNEL staining. Middle and bottom rows: Red, Iba-1; blue, DAPI; scale bar: 20 μ m. (B) ZO-1 staining (the outer limiting membrane) at each indicated weeks of age. The stain was a clear solid line in the wild-type retina but was fragmented in *rd* retina at 2, 3, and 13 weeks (arrowheads). The line was even fainter at weeks 4 and 8 (arrows). Red, ZO-1; blue, DAPI; scale bar: 20 μ m. (C) GFAP expression in the course of retinal degeneration in *rd* mice. Red, GFAP; blue, DAPI; scale bar: 20 μ m. Abbreviations: INL, inner nuclear layer; ONL, outer nuclear layer.

Figure 1. Continued

significantly better cell survival with neurite extension when the cells were transplanted on the fifth or sixth week after the period of massive rod loss with active microglial accumulation (Fig. 4A, B in controls, $p < 0.05$). ChABC application during rod loss had no effect, but it significantly increased the integration of graft cells during the later stages, such as postnatal weeks 6 and 8 ($p < 0.01$, unpaired t test).

Effect of VPA on Cell Transplantation

We transplanted retinal cells in the presence of different concentrations of VPA in 6-week-old *rd* mice. Because we observed the most efficient graft cell integration at 1 mM VPA, this concentration was used for all subsequent experiments (Fig. 5A).

When we transplanted cells into *rd* mice at different postnatal weeks in the presence of VPA, we tended to observe a larger number of integrated cells in the earlier transplantations, and we observed a significant difference when cells were transplanted during the fourth week ($p < 0.01$, unpaired t test) (Fig. 5B). This suggests that the host environment is more able to accept replaced cells for the lost ones. Since VPA was observed to enhance rod differentiation (24), we tested if the treatment of VPA enhanced rod differentiation in these graft cells. The transplanted retina was studied on days 4, 5, 6, and 10 after the transplantation to evaluate the percentage of rhodopsin-positive cells among Nrl-GFP cells. The percentage was consistently higher in VPA-treated grafts throughout the observed period (Fig. 5C). With the treatment of VPA, the graft cells survived for at least 3 months without a significant time-dependent loss (Fig. 5D).

Effect of Glatiramer Acetate on Cell Transplantation

In this experiment, 1 mM VPA was routinely added to the cell suspension for transplantation. Preimmunization with glatiramer acetate (GA) increased the number of integrated cells, and a significant difference was observed during transplantations in the fourth postnatal week ($p < 0.01$, unpaired t test) (Fig. 4B). Graft cells poorly survived when transplanted in postnatal 2-week mice with or without glatiramer acetate.

DISCUSSION

Transplanted retinal cells reportedly integrate into the host retina of wild-type mice or into the remaining structure of the ONL in degenerating mice (14,16). Once integrated into the ONL, the graft cells exhibit photoreceptor morphology indistinguishable from the neighboring photoreceptors of the host retina. These integrated cells were also reported to function as photoreceptors. However, the first clinical application of retinal cell transplantation would be in those cases with no functional ONL (i.e., photoreceptors). Even though *rd* mice generally show rapid degeneration and marked gliosis, a number of reports showed that the remaining neurons retain function to some extent (3,18) and some electron micrographs or superior colliculus recordings showed evidence that retinal sheets transplanted in *rd* mice make synaptic connections if the transplantation was performed while *rd* mice were young (3,8,26). In our experiments using dissociated cell transplantation, we have not proven a synaptic connection yet, but many of these cells were mechanically attached to the host retina, indicating some interaction between the graft and the host. These cells were also

Figure 2. Secondary changes in the number of other retinal cells. (A–D) Red, rod bipolar cells (PKC); blue, DAPI; scale bar: 20 μ m. (E and F) PKC staining (green) on whole-mounted retina from an 11-week-old *rd* mouse with positioning central to peripheral from right to left. Scale bar: 50 μ m. (E) Peripheral area. (F) Central area. (G and H) PNA labeling of cone outer segments (red) in whole-mounted retina from a 7-week-old *rd* mouse with positioning central to peripheral from right to left. Scale bar: 10 μ m. (G) Peripheral area. (H) Central area. (I) The number of PKC-positive cells in the central, midperipheral, and peripheral retina was counted on the whole-mounted staining (mean $\pm$ SEM). (J) The number of PNA-labeled cells (outer segments) in the central, midperipheral, and peripheral retina was counted on the whole-mounted staining (mean $\pm$ SEM). Abbreviations: ONL, outer nuclear layer; INL, inner nuclear layer.

found next to PKC-positive rod bipolar cells, suggesting a possible synaptic connection. In the wild-type retina, dissociated photoreceptors were well incorporated, but whether the sheet implantation is better than dissociated cell implantation or we need more number of cells to integrate in order to evoke host light perception is yet to be tested in the future. Also, the presence of outer segments with dense rhodopsin accumulation is an important factor for sensitive light perception. Even though transplanted photoreceptors would not form proper outer segments,

these cells often had condensed rhodopsin inner segment-like outgrowths in our transplantation. Ganglion light responses were recorded in rat retinal degeneration models with no outer segments (2), and our graft cells with accumulated rhodopsin outgrowths would give a chance for light perception, even though they may not be as sensitive as transplanted retinal sheets with outer segments. Our study at least showed that cell survival and mechanical interaction of host graft cells could be definitely improved by selecting a proper time window

Figure 3. (A, B) Integration of graft cells into wild-type host retina. Nrl-GFP cells (p4) were transplanted into 6-week-old wild-type B16 mice. (A) Integrated graft cells were indistinguishable from host photoreceptors unless they were immunostained. Red, GFP; scale bar: 10 μ m. (B) Graft cells have Bassoon-positive synaptic terminals (arrows), and many of them reach the proper outer plexiform layer (arrowhead). Green, GFP; red, Bassoon. (C) In *rd* mice, most of the graft cells and the Bassoon-positive axon terminals (arrows) remained outside of the row of cones (arrowheads). Green, GFP; red, Bassoon. Scale bar: 10 μ m. (D) PKC-positive bipolar dendrites and graft cells came into close proximity of each other (arrows). Green, GFP; red, PKC. Scale bar: 10 μ m. (E, F) One month after transplantation of Nrl-GFP retinal cells in a 6-week-old *rd* mouse. GFP-positive cells had typical photoreceptor nucleus (E), and they are aligned well against PKC-positive cells (F). Green, GFP; red, PKC. Scale bar: 10 μ m. (G, H) Two months after transplantation of Nrl-GFP retinal cells in a 4-week-old *rd* mouse. Graft cells extend neuritis on host retina (arrow), and these cells often accumulate rhodopsin at its inner segments like outgrowths (arrowheads). Green, GFP; red, rhodopsin. Abbreviations: ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; OS, outer segment.

for transplantation and by using some additional factors to modulate the host environment with the progressive retina even with the “ONL lost model.”

We have established a protocol for photoreceptor differentiation and semipurification from embryonic stem (ES) or induced pluripotent stem (iPS) cells of mice, monkeys, and humans (9,17,22,23), and more recently, a

three-dimensional retinal sheet was successfully differentiated from mouse ES cells (6). With the advance in such practical graft cell preparation, a strategy to optimize conditions of cell-based therapy would be essential for the first lines of patients where retina may be severely damaged.

We first studied the background features associated with the course of rod degeneration in *rd* mice. Microglia

Figure 4. (A) The number of integrated cells at different transplantation times with or without ChABC. The cell number represents the total number of graft cells with neurite extension in every 50- μ m section (mean $\pm$ SEM; $p < 0.01$, unpaired t test). (B) The number of integrated cells at different transplantation times with or without preimmunization of glatiramer acetate (GA). The cell number represents the total number of graft cells with neurite extension in every 50- μ m section (mean $\pm$ SEM; $p < 0.01$, unpaired t test). VPA (1 mM) was added to graft cell suspension in this experiment.

Figure 5. The effect of VPA on graft cell integration. (A) Different concentrations of VPA were added to the graft cell suspension at the time of transplantation (mean $\pm$ SEM). (B) Cell integration was evaluated with 1 mM VPA at different transplantation times (mean $\pm$ SEM; $p < 0.01$, unpaired t test). (C) Time-dependent rhodopsin-positive percentages among Nrl-GFP-positive cells were evaluated after transplantation. (D) Numbers of surviving graft cells attached to host retina after transplantation.

accumulation and GFAP expression were enhanced during the second postnatal week when massive rod cell death was in progress as evidenced by a large amount of TUNEL-positive cells in the ONL (Fig. 1). Although the amounts of microglia and GFAP expression once decreased after rods were lost, GFAP expression again became stronger in later weeks. This transient activation of GFAP at the time of rod death was also observed previously (29) and could be distinguished from typical and permanent (~8 weeks) gliosis, which is known to inhibit transplant entry in later phase of degeneration (8). Interestingly, the transient increase in ramified microglia in the IPL was observed during the third week, possibly indicating some synaptic modification in the second to third neurons that was previously reported as changes in the inner retina in the early phase (29). The outer limiting membrane is another factor thought to inhibit graft cell entry (25). In *rd* mice, the ZO-1 staining that labels the outer limiting membrane was fragmented during the time of progressive rod death and was faint at 4 and 8 postnatal weeks (Fig. 1). Cones and rod bipolar cells were also gradually lost after rod death from the central retina, which is consistent with a previous report (15). Taken together, these results suggest that cell transplantation should be reasonably efficient during after the rod loss period but before the increased GFAP expression and/or the secondary loss of other neurons. In *rd* mice, this specific period was estimated to be from the fourth to eighth postnatal weeks.

We observed better graft cell survival with the host when we transplanted cells on the fifth to sixth week of *rd* mice compared with those transplanted at the fourth week or earlier (Fig. 4), supporting our assumption that cells do not efficiently survive the period of massive rod loss when a large number of dying cells are intensively removed by microglia.

ChABC treatment significantly enhanced cell integration with the transplantation during the sixth and eighth weeks, indicating that gliosis begins around the sixth week. Conversely, ChABC treatment exacerbated microglia invasion into the host retina with transplantation in the second week (data not shown), suggesting that CSPGs have a protective effect against inflammatory infiltration in the earlier stage of degeneration. In contrast to the advantage of using ChABC at a later stage, the statistically significant effects of VPA and glatiramer acetate on transplantation were observed during the earlier period, such as the fourth week. The effect of glatiramer acetate suggests that the inflammatory environment is more easily induced immediately after the period of massive rod loss, which is partly due to the large number of accumulated microglia for dying rods that may be easily activated by the surgical procedure. The loss of graft cells during the first month reportedly depends on the surgical injury (32). The effect of VPA during the fourth week

indicates that the neurons remaining in the host retina are in a ready state to accept lost first neurons and that the more efficient differentiation of graft cells facilitates synapse formation and survival in these cells. Whether these graft cells formed functional synapses with the host cells and whether VPA actually affected synapse formation are yet to be proven. Nevertheless, these data taken together indicate that graft cells have a better chance of survival and possible connection to the degenerating host if they are transplanted at the right time, even after complete rod loss.

Retinitis pigmentosa (RP), a heterogeneous group of inherited retinal disorders characterized by progressive photoreceptor apoptosis, has more than 40 different causal genes (10). This degenerative process generally takes decades and therefore different stages of secondary neuron loss or gliosis may exist in the same retina (21,27). The speed and the process of degeneration vary depending on the causal genes. Therefore, a detailed understanding of the disease progress in each patient or in each genetic type is essential, and patients may have a considerably long time window for adequate transplantation if the segment of transplantation is properly selected within the retina.

In conclusion, we observed that the transplanted retinal cells survived well with neurite extension into the host retina with retinal degeneration when transplanted at the right time, even after the ONL loss. These graft cells survived better with the help of some additional factors such as ChABC, glatiramer acetate, and VPA. Our results will help optimize transplantation protocols as to the timing and strategies in considering the clinical application of cell-based therapy for retinal degeneration diseases.

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