Article

A Subsequent Human Neural Progenitor Transplant into the Degenerate Retina Does Not Compromise Initial Graft Survival or Therapeutic Efficacy

Bin Lu¹, Yanhua Lin¹, Yuchun Tsai¹, Sergey Girman¹, Grazyna Adamus², Melissa K. Jones¹, Brandon Shelley¹, Clive N. Svendsen¹, and Shaomei Wang¹

¹ Board of Governors Regenerative Medicine Institute, Cedars-Sinai Medical Center, Los Angeles, CA, USA

² Casey Eye Institute, OHSU, Portland, OR, USA

Correspondence: Shaomei Wang, Board of Governors Regenerative Medicine Institute, Cedars-Sinai Medical Center, Los Angeles, CA 90048, USA; e-mail: shaomei.wang@cshs.org

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Purpose: Stem and progenitor cell transplantation provides a promising clinical application for treating degenerative retinal diseases, including age-related macular degeneration (AMD) and retinitis pigmentosa (RP). Our previous studies have shown that a single subretinal injection of human cortical-derived neural progenitor cells (hNPC^{ctx}) into cyclosporine-treated Royal College of Surgeons (RCS) rats preserved both photoreceptors and visual function. However, it is still unknown whether nonautologous progenitor cell readministration for sustained vision is efficacious and safe in terms of the initial graft initiating an immune response to a subsequent graft.

Methods: A cell suspension containing 3×10^4 hNPC^{ctx} into one eye of cyclosporine-treated RCS rats at postnatal day 21 (P21), followed by a second transplantation at P95 into the previously untreated fellow eye.

Results: hNPC^{ctx} delayed photoreceptor degeneration and preserved visual function, as measured by electroretinography (ERG), optokinetic response (OKR), and luminance threshold recordings (LTRs). Visual function and photoreceptors of the initially treated eye were still preserved 6 weeks after hNPC^{ctx} were injected into the second eye. Antibodies against T-cell markers showed that CD3, CD4, and CD8 T cells were not detected at P90 and P140 in most cases. No detectable level of anti-nestin antibody was found in serum by enzyme-linked immunosorbent assay (ELISA).

Conclusions: This xenograft study with cyclosporine-treated animals demonstrates that readministration of hNPC^{ctx} into the fellow eye did not induce anti-graft immune responses or lower therapeutic efficacy of hNPC^{ctx} in preserving vision. Thus, readministration of progenitor cells to sustain long-term efficacy may be an option for long-term therapies of retinal degeneration.

Translational Relevance: Redosing neural progenitors do not affect the efficacy of the initial grafts in protecting vision or induce unwanted immune responses.

Introduction

Retinal degenerative diseases, such as age-related macular degeneration (AMD) and retinitis pigmentosa (RP), which are characterized by photoreceptor loss and visual dysfunction, are largely untreatable conditions and affect millions worldwide.^{1–7} Recent advances in gene therapy offer real hope to treat some of these diseases. Gene therapy for the RPE65 mutation, which is associated with Leber's congenital amaurosis type 2, has been used in the clinic.^{8–11}

However, specific genetic defects with single gene mutation have been found in only a few patients, thereby limiting the potential application of gene therapy. Furthermore, gene therapy improves vision, but does not slow the progression of photoreceptor degeneration. A generic blanket therapy for all retinal dystrophies may be a better global strategy.

Stem/progenitor cells offer real potential for regenerative medicine to treat a variety of diseases, including retinal degeneration.^{12–19} Among the many types of stem/progenitor cells, human neural pro-

genitor cells (hNPCs) from the forebrain cortex (hNPC^{ctx}) have been demonstrated to be a good cell source for treating retinal degeneration. We have shown that a single subretinal injection of hNPC^{ctx} leads to their migration and long-term engraftment in the subretinal space, as well as preserves vision in Royal College of Surgeons (RCS) rats, a well-established animal model for retinal degeneration.^{20,21} This ability to survive in the subretinal space and offer neuroprotection is a powerful feature of these cells in treating retinal degenerative diseases. In addition, their ability to migrate long distances after subretinal injection permits the injection site to be targeted at the transient zone to avoid damaging macular vision that is already fragile from degeneration. Finally, hNPC^{ctx} do not compromise normal retinal function after subretinal transplantation into nonhuman primates²² and do not appear to form tumors following transplantation in the central nervous system (CNS).^{23–28}

While there are several clinical trials using stem/progenitor cells to treat retinal degeneration (ClinicalTrials.gov), some fundamental questions remain to be addressed. Stem cells are among the most complex biological therapeutic entities proposed for clinical use.¹⁹ Before stem cell therapy becomes part of a standard clinical practice, safety issues, graft survival, long-term efficacy, immune rejection of allogeneic cells, and the feasibility for multiple treatments must be resolved in order to ensure their benefit for humans. The advantage of grafting stem cells into the subretinal space/vitreous cavity is that both are immune privileged,^{29,30} which means grafts are more likely to survive by avoiding a host immune response. However, this immune privilege may be lost in the degenerative retinal condition or when the outer blood-retinal barrier is disrupted.^{31,32} Indeed, retinal pigment epithelium (RPE) cells that were normally major histocompatibility complex (MHC) class II-negative expressed MHC class II mRNA after injection into the subretinal space of RCS rats and the grafts with disparity at MHC class I and class II were rejected.³¹ Not only can a compromised host environment lead to an increased immune response, but also the integrity of donor cells affects the host immune response. For instance, human embryonic stem cells (hESCs) express human leukocyte markers (HLA) that mediate immune responses, thus making hESC-derived RPE grafts more susceptible to rejection.³³ In addition, MHC classes I and II proteins are present on grafted fragmented retinal tissue, but not on the retinal whole sheet.³⁴ Syngeneic Schwann cells

continue to preserve vision after subretinal injection into RCS rats, while the efficacy of allogeneic Schwann cells deteriorated with time.³⁵ Finally, mouse postmitotic rod photoreceptor cells with partially mismatched H-2 haplotypes to the host were significantly reduced 4 months after subretinal injection, with macrophages and T cells present around the graft site indicating a chronic immune response.³⁶ In contrast, engrafted neural progenitor cells have been shown extensively by our laboratory and others to have robust and long-term survival in several animal species, to promote recovery in various models of neurodegenerative diseases^{37–40} and are already being used in clinical trials (NCT01632527).

Treating neurodegenerative diseases by completely halting the ongoing degeneration is the ultimate goal, but this is generally not yet attainable. An alternative may be to readminister stem/progenitor cells to ensure their long-term efficacy in treating degenerative diseases, for instance in retinal degeneration. To date, no animal study of retinal degeneration has shown whether the host tolerates additional subretinal injections and how the second injection of stem/progenitor cells into the previously untreated eye affects the effectiveness of the first injection of stem/progenitor cells on preserving vision. In addition, it is important to determine how the host immune system responds to stem/progenitor cells that may enter into the blood stream via the choroid during injection. The main goal of this study is to address the above questions by engrafting hNPC^{ctx} at two separate time points into cyclosporine-treated RCS rats. Ideally this double xenograft model will not elicit a significant host immune response, but will instead confirm and even prolong the preservation of vision shown in our previous single injection study.^{21,20} If so, the great potential of hNPC^{ctx} to treat retinal degeneration may permit translation from current animal models to clinical trials in the near future.

Materials and Methods

Animals

RCS rats were grouped as follows: Group 1: RCS rats received subretinal injections of hNPC^{ctx} ($n = 10$) at postnatal day 21 (P21); RCS rats received balance salt solution (BSS) ($n = 8$) at P21; untreated RCS rats ($n = 13$) and untreated Long Evans rats ($n = 3$); Group 2: RCS rats ($n = 11$) received the first subretinal injections of hNPC^{ctx} at P21, and the second injection

of hNPC^{ctx} ($n = 8$) or BSS ($n = 3$) into the fellow eye at P95.

Rats received a single injection at P21 and were euthanized at P90 or at P140. Additional rats received a second injection into the fellow eye at P95 and were euthanized at P140. Rats received dexamethasone intraperitoneally for 2 weeks (2.5 mg/kg/day) after the first subretinal injection of hNPC^{ctx} and the immunosuppressant cyclosporine A (CyA; Novartis, Basel, Switzerland) in the drinking water (210 mg/L; resultant blood concentration of 300 µg/L)⁴¹ from 1 day before transplantation until sacrifice. At 1 to 5 weeks after the second injection, blood was collected for antibody analysis from all treated and untreated rats. These studies were conducted with the approval and under the supervision of the Institutional Animal Care Committee at the Cedars-Sinai Medical Center. All animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

hNPC^{ctx} Derivation and Expansion

Cortical tissue was collected from an 8-week-old postmortem fetal tissue by Guido Nikkhah (Germany) with institutional review board approval by his institution and by the University of Wisconsin-Madison, where the resulting cell line was generated. The intact primary cortical mantle was identified, isolated, and dissociated into a single cell suspension. The detailed protocol was described in Shelley et al.⁴² Vials of the Master Cell Bank were thawed in Clive Svendsen's research laboratory at Cedars-Sinai Medical Center, and cells were expanded once more using the current good manufacturing practice and standard operating procedures to produce a research-grade Working Cell Bank at passage 24 for transplantation studies.

Transplantation

Aliquots of hNPC^{ctx} were stored in liquid nitrogen and upon thawing were washed with BSS. A cell suspension containing 3×10^4 cells/eye in 2 µL of BSS carrying medium was delivered into the subretinal space through a small scleral incision with a fine glass pipette (internal diameter, 75–150 µm) attached by tubing to a 25-µL syringe (Hamilton, Reno, NV). The cornea was punctured to reduce intraocular pressure and to limit the efflux of cells. A sham-surgery group was treated with carrying medium alone. Immediately after injection, the fundus was examined for retinal damage or signs of vascular distress. Any animals

showing such problems were excluded from further study.

Optokinetic Response (OKR)

Animals were tested for spatial visual acuity using an optomotor testing apparatus (Cerebral Mechanics, Lethbridge, AB, Canada) at several time points after the second injection of hNPC^{ctx} or BSS into the fellow eye. The optometry set-up comprises four computer monitors arranged in a square that project a virtual three-dimensional (3-D) space of a rotating cylinder lined with a vertical sine wave grating. An unrestrained rat was placed on a platform in the center of the square to track the grating with reflexive head movements. The spatial frequency of the grating was clamped at the viewing position by repeatedly recentering the "cylinder" on the head of the test subject. Acuity was quantified by increasing the spatial frequency of the grating using a psychophysics staircase progression until the optokinetic reflex was no longer elicited, thereby obtaining a maximum threshold.

Electroretinography (ERG)

Before ERG was conducted, animals were dark-adapted for 15 hours in complete darkness. Rats were then anesthetized with an intraperitoneal injection of 25 mg/kg ketamine and 2 mg/kg dexmedetomidine. Scotopic ERG was recorded binocularly with monopolar contact lens electrodes using a Diagnosys system with ColorDome stimulator and Espion software (Diagnosys LLC, Lowell, MA). Signals were amplified and band pass-filtered between 0.3 and 150 Hz. The stimulus parameters were as follows: duration, 5 ms; brightness, 25 cd/m²; and interstimulus intervals, 10 seconds. ERGs were averaged for 10 stimulus repetitions.

Luminance Threshold Recordings (LTR)

The functional state of the retina was evaluated by recording the multineuronal responses in multiple (16–18) microelectrode penetrations into the unilateral superior colliculus of anesthetized rats. At each recording site, the receptive field was located by presenting flashes of the light spot of 3° in diameter. Response luminance threshold was then measured and defined as a minimal luminance of the stimulating light spot eliciting criterion multiunit response (of amplitude twice of the level of the background activity). This procedure results in a map of focal luminance thresholds over the whole visual field of

the eye contralateral to the tested superior colliculus, and provides a topographic indication of the magnitude and area of photoreceptor rescue across the retina. Based on these recordings, the cumulative curve of the luminance thresholds across the retina was calculated, which showed the percent of retinal area (y-axis) where the visual thresholds were less than the values indicated on the x-axis.

Enzyme-Linked Immunosorbent Assay (ELISA)

Blood was collected via tail vein at 1 week, 2 weeks, and 4 to 5 weeks after the second injection of hNPC^{ctx} or BSS in the fellow eye. Sera were stored at -80°C prior to analysis for anti-human antibody. Anti-human nestin antibody was chosen since hNPC^{ctx} expressed nestin both in vitro and in vivo. An ELISA kit was used to detect anti-human nestin according to the manufacturer's instruction (NES ELISA Kit, Antibodies-online). Background readout without serum was used as negative control. Sera were diluted with phosphate buffered saline (PBS) into 1:2 and incubated for 2 hours, followed by incubation with biotin-labeled secondary antibodies for 1 hour, then with a Horse Radish Peroxidase-conjugated avidin antibody for an additional hour. After washing, 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was added for 30 minutes. The reaction was terminated by stop solution, and optical density was determined using a microplate reader at the wavelength of 450 nm. All incubations were done at 37°C .

Histology

At the end of experiment, eyes and spleens were removed and immersed in 2% paraformaldehyde for 1 hour, then infiltrated with 30% sucrose and embedded in optimal cutting temperature compound (OCT). Horizontal 10- μm sections were cut on a Leica cryostat. Four sections (50 μm apart)/slide were collected in five series. One set of serial cryosections was stained with cresyl violet, and the remaining sections were used for immunohistochemistry. Finally, sections were examined with a regular brightfield microscope and a computer-based image: ProgRes capture system (Jenoptik, Jupiter, FL) or a Nikon C2⁺ confocal microscope (Nikon Instruments Inc., Melville, NY).

Immunohistochemistry

Retinal sections were permeabilized and blocked with 0.25% Triton X-100 + 5% horse serum for 1 hour

at room temperature (RT). then incubated with primary antibodies (see below) or PBS alone overnight at 4°C . The next day, sections were washed with PBS and labeled with anti-mouse or rabbit secondary antibodies conjugated with Alexa Fluor-488 or Alexa Fluor-568 (Life Technologies, Grand Island, NY) for 1 hour at RT and counter-stained with 49,69-diamidino-2-phenylindole (DAPI). Primary antibodies against the following proteins were used: Recoverin (rabbit, 1:2000; Millipore, Billerica, MA), Arrestin (rabbit, 1:1000; Millipore), human nuclear marker, MAB1281 (mouse, 1:300; Millipore), Ki67 (rabbit, 1:500; Millipore), Nestin (rabbit, 1:2000; Millipore), glial fibrillary acidic protein (GFAP; rabbit, 1:1000; Sigma, Sigma-Aldrich, St. Louis, MO), CD3 (1:1000), CD4 (1:100), and CD8 (1:200; CD3, CD4, and CD8 are all anti-rat, BD Biosciences, San Jose, CA). Images were taken with the confocal microscope (Eclipse C1si; Nikon Instruments, Inc.).

Statistical Analysis

Data were analyzed with GraphPad Prism 5.01 for Windows (GraphPad Software, Inc.). Statistical analyses were made using analysis of variance (ANOVA) and Newman-Keuls multiple comparison test. Data were expressed as mean $\pm$ SEM, and differences were considered to be significant at $P < 0.05$.

Results

A single graft of hNPC^{ctx} has been shown to protect photoreceptors and to have a significant functional affect in RCS rats. However, certain retinal degenerative diseases may benefit from repeat hNPC^{ctx} delivery. In order to test the effect on therapeutic efficacy of readministration of human neural progenitors into the degenerate retina, this study transplanted hNPC^{ctx} into the RCS rat at P21 and then transplanted hNPC^{ctx} into the fellow eye at P95. As this repeat cell delivery has not been addressed in retinal degeneration, it was critical to ensure that readministration of hNPC^{ctx} to the fellow untreated eye is tolerated by the host immune system and does not affect the survival or function of the initial hNPC^{ctx} graft.

Immune Response after Subretinal Injection of hNPC^{ctx}

To verify that readministration of hNPC^{ctx} to the fellow untreated eye is tolerated by the host, we examined the immune response in the RCS rats after a

Figure 1. Analysis of CD3, CD4, and CD8 T cells in the retina originally treated with hNPC^{ctx} before and after subretinal injection into the fellow eye. Retinas treated with hNPC^{ctx} at P21 were collected at P90 (A–C) and P140 (D–F). Additionally, treated retinas were collected at P140 after the fellow eye received hNPC^{ctx} at P95 (G–I). Cryosections immunostained with antibodies against CD3, CD4, and CD8 T cells (green) and stained with DAPI (blue) showed no infiltrating cells, with only background staining of the debris layer. INL, inner nuclear layer; RGC, retinal ganglion cell layer. Scale bar = 100 μ m.

single hNPC^{ctx} and a subsequent hNPC^{ctx} grafts. We used the T-cell markers CD3, CD4, and CD8 in order to confirm that repeat hNPC^{ctx} injections do not initiate host adaptive T-cell activation. The retina receiving a single graft at P21 showed no positive staining for CD3, CD4, and CD8 at P90 (Figs. 1A–C) or P140 (Figs. 1D–F). Retinal sections of treated eyes after a second injection of hNPC^{ctx} or BSS into the fellow eye at P95 also showed no presence of CD3,

CD4, and CD8 T cells (Figs. 1G–I) at P140. Positive staining for CD3, CD4, and CD8 in spleen sections confirmed proper antibody function (Supplementary Figs. S1A–C). It must be noted that 1 of 13 rats receiving a hNPC^{ctx} injection at P21 and 1 of 8 rats receiving a hNPC^{ctx} injection at P95 in the fellow eye showed the presence of numerous CD3, CD4, and CD8 T cells in the inner retina and debris layer (Figs. 2A–C). The reaction was mostly around the graft

Retina at P140 with 1st hNPC^{ctx} at P21 and 2nd hNPC^{ctx} at P95 into fellow eye

Figure 2. T-cell presence in the eye after subretinal injection of hNPC^{ctx} in 2 out of 18 examined eyes. Retinal cryosections from the treated eye were immunostained with antibodies against CD3, CD4, and CD8 T-cells (green) and stained with DAPI (blue). (A–C) Scattered positively stained T-cells were detected in the choroid and inner retina of the treated eye at P140 after grafting of hNPC^{ctx} in the fellow eye. Scale bar = 100 μ m.

Figure 3. ELISA quantification of anti-human nestin antibody after initial and subsequent subretinal injection of hNPC^{ctx}. Background readout without serum was used as negative control. Sera collected from rats receiving initial grafts at P21 and assessed at P90 and P140 and rats receiving subsequent grafts at P95 and assessed at 8 days, 2 weeks, and 4 to 5 weeks post the second injection into the fellow eye. The optical density values were similar to that of the negative control, indicating there is no presence of anti-human nestin antibody in the blood of rats after single or repeat subretinal hNPC^{ctx} injections. Data were expressed as mean $\pm$ SEM.

area, suggesting that the inflammation was likely due to the initial mechanical injection, which highlights the importance of examining the fundus immediately after injection for retinal damage or signs of vascular distress. It is possible that there was slight damage in these two outliers that led to their uncharacteristic immune response. Further examination showed that both photoreceptors and visual function were not comprised in these rats. We also tested whether transplanted rats produced specific antibodies against the hNPC^{ctx}, by assessing human-specific nestin that we previously showed is expressed in a high proportion of transplanted hNPC^{ctx}.²⁰ Rat sera collected weekly and tested by ELISA showed nestin levels were similar in negative controls (Background readout without serum) and rats receiving a single injection of hNPC^{ctx}. In addition, the levels of antibodies against nestin following a repeat injection into the fellow eye were the same as control and single injection levels (Fig. 3).

Overall, the majority of animals had no T-cell marker expression and no single animal produced antibodies against nestin following either a single or double xenografts. These findings show that the first subretinal graft is not sensitizing the host to mount an immune response to subsequent grafts and thereby suggest that multiple injections may be feasible in future clinical trials.

Figure 4. Confocal analysis of retinal morphology and transplant phenotype after subretinal hNPC^{ctx} injection. (A) Retinal cryosection stained with HuNu (green, arrows) and DAPI (blue) shows that injected hNPC^{ctx} were located between the RPE and ONL in the subretinal space (debris layer). (B) Cone photoreceptor cells, labeled with an antibody against arrestin (red), showed a fairly normal morphology in the treated retina (double arrows). (C, D) Immunolabeling hNPC^{ctx}-treated retinas with antibodies against recoverin (red) and HuNu (green, arrows) showed no overlapping label between photoreceptor cells and hNPC, suggesting that hNPC^{ctx} were not differentiating into photoreceptor cells. (E) Immunostaining the treated retina with antibodies against vimentin (red) and GFAP (green), both specific Müller glial cell markers, showed that donor cells only expressed GFAP. (F) Immunostaining the treated retina with antibodies against human nestin (green) and Ki67 (red) showed only a few Ki67-positive cells within the grafts (arrows). Scale bar = 100 μ m.

hNPC Transplant Survival, Distribution, and Phenotype

Cell survival, migration, and phenotype are all factors that can greatly influence graft function.

Hence, it was important to characterize the transplant distribution and composition required to preserve photoreceptors and ultimately vision. Human-specific antibodies, used to detect the human transplant in the rat retina, showed that hNPC^{ctx}

survived up to 120 days posttransplantation and migrated between the RPE and photoreceptors (Fig. 4A, 4D). Further, the morphology of cone photoreceptors was well preserved as evident from a cone-arrestin antibody that labeled nearly normal-appearing cone photoreceptors with outer and inner segments and synaptic endings in graft-protected area (Fig. 4B). To examine whether transplanted hNPC^{ctx} differentiate into retinal neurons, retinal sections were double stained with anti-human nuclear marker (HuNu) and an antibody against recoverin (photoreceptor and On-cone bipolar cell marker). Colocalization of recoverin and HuNu was never detected at both P90 and P140 time points (Figs. 4C, 4D). The lack of retinal differentiation is to be expected as these cortical-derived progenitor cells are biased towards a neural phenotype and cell transplants have consistently been shown to express nestin (a neural progenitor marker) and to a lesser degree GFAP (a glial cell marker) and vimentin (a Müller glia cell).²⁰ Indeed, hNPC^{ctx} transplants in the retina were shown to express GFAP (Fig. 4E) and did not express vimentin, a marker for retinal Müller glial cells. One of the major concerns of stem cell therapy is tumor formation after grafting. Compared to stem cells, progenitor cells have the benefit of reduced proliferative capacity, and consequently no tumor formation has been seen after extensive use of hNPC^{ctx} in various animal models.^{23–26,28} Ki67 (a proliferation marker) was expressed at only low numbers in the nestin-positive human progenitor cells (Fig. 4F), confirming that the majority of hNPC^{ctx} ceases to proliferate following retinal transplantation. Importantly, there was no evidence of uncontrolled overgrowth even at P140, suggesting that these cells are safe for repeat and long-term transplantation.

hNPC^{ctx} Transplants Continue to Preserve Photoreceptors after Subsequent hNPC^{ctx} Transplantation

The outer nuclear layer (ONL) of the healthy retina is comprised of photoreceptor cells aligned in approximately 10 to 12 cell layers. In RCS rats, the ONL is lost due to continued degeneration of these photoreceptor cells. We transplanted human cortical-derived progenitor cells into one eye to confirm our previous report that hNPC^{ctx} grafts can protect photoreceptor cell degeneration. Subsequently, we engrafted hNPC^{ctx} into the previously untreated eye

to test whether this second engraftment would affect the original preservation of photoreceptors.

General photoreceptor survival and retinal lamination in the RCS rats was examined by cresyl violet staining on retinal sections from untreated control, BSS-sham, and cell-treated animals at P90 and P140. Consistent with our previous study, the ONL of the untreated eye was reduced to one cell layer at P90 (Fig. 5A), and further reduced to a sparsely distributed nuclear layer at P140, the end time point in the current experiment (data not shown). Similarly, the sham retina injected with BSS at P21 also showed one layer of ONL at P90 (Fig. 5B). Importantly, subretinal injection of hNPC^{ctx} at P21 preserved photoreceptors, showing six to eight cell layers in the ONL at P90 (Fig. 5C), and still five to seven cell layers in the graft-protected area at P140 (Fig. 5D). The fellow eye that received hNPC^{ctx} at only P95 had just one layer of cells in the ONL at P140 (Fig. 5E). At the same P140 time point, the ONL of the eye originally treated at P21 with hNPC^{ctx} still had five to seven layers of cells after the fellow eye received the second injection of hNPC^{ctx} at P95 (Fig. 5F). This finding confirms that hNPC^{ctx} can protect degenerating photoreceptors and suggests that a second injection of hNPC^{ctx} in the untreated fellow eye has no adverse effect on the efficacy of hNPC^{ctx} in preserving photoreceptor survival in the first treated eye.

hNPC^{ctx} Transplants Continue to Preserve Visual Function after Subsequent hNPC^{ctx} Transplantation

The retinal degeneration in RCS rats leads to blindness by 150 days if left untreated. While engrafted hNPC^{ctx} clearly protected photoreceptors and this was maintained following a repeat injection, we still needed to confirm that this photoreceptor protection led to a preservation of vision. We performed three functional tests on RCS rats after the initial and subsequent subretinal hNPC^{ctx} injections. OKR offers noninvasive screening to detect visual acuity, but it is not a sensitive measurement to correlate photoreceptor thickness and visual acuity. ERG provides a gross measure of retinal activity and indicates the relative function of rods and cones; however, it is not sensitive enough to detect local subretinal effects. LTRs from the superior colliculus measures the sensitivity to light stimuli across the visual field, which in turn provides a geographic indication of the magnitude and area of photorecep-

Figure 5. Effect of hNPC^{ctx} grafts on photoreceptor protection in RCS rats. Cresyl violet staining showed only one layer of photoreceptors was present at P90 in the untreated retina (A) and in the BSS-treated retina (B). Treated eyes receiving hNPC^{ctx} at P21 had five to seven layers of photoreceptors in the grafted area (arrows: grafted hNPC^{ctx}) at P90 (C) and P140 (D). Fellow eyes received hNPC^{ctx} (arrows: grafted hNPC^{ctx}) at P95 showed one layer of photoreceptors at P140 (E). Treated eyes had five to seven layers of photoreceptors in the grafted area (arrows: grafted hNPC^{ctx}) at P140 after fellow eyes received hNPC^{ctx} at P95 (F). Scale bar = 100 μ m.

tor rescue across the retina.^{21,43} The LTRs can specifically detect graft-related effects.

Visual acuity, measured by OKR, was 0.35 ± 0.01 and 0.29 ± 0.01 cycle/degree (c/d) in the untreated RCS rats at P90 and P135, respectively (Fig. 6A). Statistical analysis revealed that rats receiving BSS injection as a control at P21 showed similar results to untreated control rats at P90 (Fig. 6A). However, a single subretinal injection of hNPC^{ctx} at P21 sustained visual acuities of 0.55 ± 0.01 (c/d) at P90 and 0.54 ± 0.01 (c/d) at P135, which was statistically greater than the untreated or BSS-injected groups at P90 and P135 ($P < 0.001$) (Fig. 6A). This confirms that hNPC^{ctx} can preserve vision after injection into a single eye. Next, we examined whether visual acuity of the eye treated at P21 is affected by a second hNPC^{ctx} subretinal injection into the fellow untreated eye, and we injected hNPC^{ctx} or BSS into the fellow untreated eye at P95. Measuring OKR at 1 week, 2 weeks, and 4

to 5 weeks after the second injection into the fellow eye showed that the visual acuity of the first treated eye was not affected by the second treatment (Fig. 6A and Supplementary Fig. S2).

ERG analysis in the scotopic-adapted untreated rats at P140 revealed that the amplitudes of a-wave and b-wave were largely lost (data not shown). In contrast, eyes treated with hNPC^{ctx} at P21 had significantly greater b-wave amplitudes at P90 compared with untreated controls ($P < 0.01$), and while b-waves were reduced in amplitude in the treated eye at P140, the b-waves were still significantly higher than the untreated eye ($P < 0.05$) (Fig. 6B). Importantly, a subsequent subretinal injection of hNPC^{ctx} or BSS in the fellow eye at P95 had no negative effect on the b-wave amplitude of the eye originally treated at P21. Indeed, the best eye had b-wave amplitude of 40 μ v after hNPC^{ctx} injection into the fellow eye, which is about 20% of normal value

Figure 6. Effect of subretinal injection of hNPC^{ctx} on visual function in RCS rats. Visual acuity of RCS rats was measured by OKR (A), ERG recordings (B), and LTRs (C). OKR measurements showed that subretinal injection of hNPC^{ctx} at P21 preserved visual acuity at normal level at both P90 (0.55 ± 0.01 c/d) and P135 (0.54 ± 0.01) time points (A), while in the untreated (Ut) eyes, visual acuity was 0.35 ± 0.01 c/d at P90 and 0.29 ± 0.01 c/d at P135. hNPC^{ctx} injection to the fellow eye at P95 exerted no adverse effect on the visual function of the treated eye at P135. ERG analysis showed that hNPC^{ctx} injection at P21 significantly preserved the amplitudes of b-wave (B) at P90. Injection of hNPC^{ctx} or BSS into the fellow eye at P95 had no adverse effect on the amplitudes of the treated eye measured at P140. (C) LTRs across the superior colliculus were performed after hNPC^{ctx} subretinal injection in the treated eyes and fellow eyes. Each curve showed the percent of retinal area (y-axis) where the visual threshold was corresponding to the value on the x-axis (log units, relative to background illumination of 0.02 cd/m^2). Data were expressed as mean $\pm$ SEM, * $P < 0.05$, ** $P < 0.01$. Note, hNPC^{ctx}-treated eyes had significantly lower luminance thresholds compared with controls, and the efficacy of hNPC^{ctx} grafted at P21 was not affected after the fellow eye received hNPC^{ctx}, BSS-injection at P95 or untreated.

(200 μV , tested in wild type rats under the same condition).

LTR analysis showed that the eye treated with hNPC^{ctx} at P21 performed significantly better than the untreated eye (Fig. 6C). Specifically, the luminance thresholds at P140 were on average 0.3 to 0.9 log units in cell-treated eyes, while age-matched untreated controls were 2.5 to 3.1 log units, indicating that the cell-treated eyes were over 100 times more sensitive to light stimuli than control eyes. It should be noted that even the most sensitive point in the RCS

rat eye, 0.3 log units, is still 10 times less sensitive to light stimuli than a wild type eye (-0.3 log units). Further analysis at P140 showed that there was no significant difference in LTRs of the originally treated eyes after the fellow eyes received injections of hNPC^{ctx} or BSS at P95. Collectively, functional data using OKR, EKG, and LTR analysis demonstrate that readministration of hNPC^{ctx} into the fellow eye had no adverse effect on the efficacy of hNPC^{ctx} preserving vision in the first treated eye.

Discussion

Stem cell therapy presents a huge potential for degenerative retinal diseases and an opportunity to replace or protect photoreceptors that are lost as a result of inherited and age-related degenerative disease.¹⁹ As retinal degeneration is an ongoing process, it is possible that clinical applications may benefit from multiple stem cell deliveries. However, a number of formidable obstacles remain to be overcome before large-scale clinical application can be effectively achieved, including immunological acceptance along with long-term engraftment, safety, and efficacy. To the best of our knowledge, the present study showed for the first time that readministration of human progenitor cells into the subretinal space of the untreated fellow eye is well tolerated by the host's immune system and, critically, does not affect the survival and efficacy of the initial graft.

Consistent with our previous studies, hNPC^{ctx} can survive for a long time and preserve photoreceptors as well as visual function.^{20,21} There were over six photoreceptor layers present at P140 in the hNPC^{ctx}-treated eye compared with only a sparsely distributed photoreceptor layer remaining in controls, and this photoreceptor preservation was unaffected by subsequent hNPC^{ctx} grafting. OKR showed normal visual acuity in cell-treated eyes compared with controls. It should be noted that injection of hNPC^{ctx} into the fellow eye at P95, when the degeneration was at the late stage, did not preserve visual function. This is to be expected, as the hNPC^{ctx} transplants are most likely not regenerating ONL once lost but, rather, they are protecting remaining ONL from degeneration. This enforces that early intervention for retinal protection is the key for successful treatment for retinal degeneration. While OKR showed normal visual acuity in cell-treated eyes, the other two tests never showed normal value in cell-treated eyes. ERG detected b-waves even at P140 in grafted eye, but at about 20% of the normal value in the best-grafted eye and LTRs showed that even the best point (lowest value) did not reach the normal retinal sensitivity to light stimulation. This is likely because the ONL was reduced to five to seven layers at P140 (compared with 10 layers in wild type retina), which highlights the importance of correlating photoreceptor preservation morphologically with functional data. We also observed that there was some donor

cell division *in vivo*; however, no uncontrolled overgrowth was detected even in long-term grafts, suggesting that the cell division is regulated.

Immune responses have been a major problem in many transplantation paradigms,^{44–50} though fortunately the eye is classically considered as an immune privileged site.^{32,51,52} Moreover, the anterior chamber-associated immune deviation (ACAID) is a form of immune tolerance due to the presence of the blood/ocular barrier, local immunosuppressive factors, and activation of some immunoregulatory T cells.^{29,53} This property of immune privilege in the eye is beneficial for cell transplantation. Progenitor cells isolated from adult and embryonic CNS^{28,54,17} hold inherent immune privilege properties, including low immunogenicity that can protect them from T-cell recognition. Consistently, long-term graft survival was seen after NPC transplantation into the eye of RCS rats up to P280,²¹ rd mice,⁵⁵ or monkeys briefly treated postsurgically with topical steroids without cyclosporine (up to 5 weeks).²²

The T cell-mediated immune response is known to play a major role in graft rejection.^{36,56–58} For example, CD8⁺ T cells are activated by an allogeneic retinal transplant,^{59–61} and CD4⁺ T cells modulate hESC immune-mediated rejection.⁶¹ Conversely, in the current study that combined the immune privilege properties of the eye and neural progenitor cells, no immune response was observed after subretinal injection in nearly all of the treated and fellow eyes. Although inflammatory T cells existed in two eyes out of 21 cell-injected eyes, we believe that this was a postsurgical complication not due to the graft itself. We did not detect anti-nestin antibodies in those animals at several time points after the second hNPC^{ctx} injection into the fellow eye, suggesting cyclosporine in drinking water was sufficient to suppress immune responses in this xenograft condition. However, as allogeneic grafts can be lost despite cyclosporine immunosuppression⁶² that inhibits T cells, we still need to investigate non-T cell-dependent responses to the hNPC^{ctx} grafts.

In summary, hNPC^{ctx} can be reintroduced into the subretinal space without diminishing the efficacy of initial stem cells in preserving vision or inducing unwanted immune responses. Thus, our results are encouraging for the repeat utilization of neural progenitor cell transplantation to treat retinal degenerative diseases.

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