

Neural Stem Cells Derived by Small Molecules Preserve Vision

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Purpose: The advances in stem cell biology hold a great potential to treat retinal degeneration. Importantly, specific cell types can be generated efficiently with small molecules and maintained stably over numerous passages. Here, we investigated whether neural stem cell (NSC) derived from human embryonic stem cells (hESC) by small molecules can preserve vision following grafting into the Royal College Surgeon (RCS) rats; a model for retinal degeneration.

Methods: A cell suspension containing 3×10^4 NSCs or NSCs labeled with green fluorescent protein (GFP) was injected into the subretinal space or the vitreous cavity of RCS rats at postnatal day (P) 22; animals injected with cell-carry medium and those left untreated were used as controls. The efficacy of treatment was evaluated by testing optokinetic response, recording luminance threshold, and examining retinal histology.

Results: NSCs offered significant preservation of both photoreceptors and visual function. The grafted NSCs survived for long term without evidence of tumor formation. Functionally, NSC treated eyes had significantly better visual acuity and lower luminance threshold than controls. Morphologically, photoreceptors and retinal connections were well preserved. There was an increase in expression of ciliary neurotrophic factor (CNTF) in Müller cells in the graft-protected retina.

Conclusions: This study reveals that NSCs derived from hESC by small molecules can survive and preserve vision for long term following subretinal transplantation in the RCS rats. These cells migrate extensively in the subretinal space and inner retina; there is no evidence of tumor formation or unwanted changes after grafting into the eyes.

Translational Relevance: The NSCs derived from hESC by small molecules can be generated efficiently and provide an unlimited supply of cells for the treatment of some forms of human outer retinal degenerative diseases. The capacity of NSCs migrating into inner retina offers a potential as a vehicle to delivery drugs/factors to treat inner retinal disorders.

Introduction

Retinal degenerative diseases such as age-related macular degeneration (AMD) and retinitis pigmentosa (RP) are the leading causes of blindness.^{1–5} These human and economic tolls will be exaggerated by the

expected doubling of prevalence by 2020 due to the aging population. The advances in stem cell technologies hold the promise of preserving vision through cellular replacement or paracrine rescue effects. Cell-based therapeutic delivery of various trophic factors protects retinal neurons from dying and stabilizes the environment observed in ischemic retinopathies, reti-

nal degenerative diseases, glaucoma, and so on.^{6–13} Preserving the existing retinal structure and visual function at an early stage of the disease with stem cells is a rational and likely successful strategy for treating retinal degenerative diseases.

For cells to be used in clinic, they must be of human origin and able to be amplified and banked; two essential prerequisites for developing viable cell-based therapies. Somatic cell nuclear transfer and induced pluripotent stem cell technology are advancing rapidly; however, a major concern and unresolved issue is the potential for tumor formation. Human embryonic stem cells (hESC) have been considered as a reliable source of specific cell types for cell-based therapy. Although specific cell types such as neural progenitor cells and/or differentiated neuronal subtypes have been routinely induced from hESC in vitro, the resulting populations were often highly mixed with different cell types and were unstable for long term culture. Moreover, the stably converted neuronal cells mostly required the integration of neuronal lineage-inducing transcription factors via viral vectors.¹⁴ Therefore, it is important to not only induce specific neuronal cell types efficiently, but also maintain their potential and characteristics stably over numerous passages under viral-free and small molecule based culture conditions. Our previous study revealed that synergistic inhibition of glycogen synthase kinase 3, transforming growth factor β , and notch signaling pathways by small molecules (CHIR 99021 and SB431542) can efficiently induce cultured hESC into homogenous neural stem cells (NSCs) under chemically defined condition.¹⁵ These NSCs can stably self renew and retain high neurogenic potential and responsiveness to instructive neural patterning cues toward midbrain and hindbrain neuronal subtypes. Previous studies have shown that neural stem/progenitor cells can protect dying neurons in the central nervous system and preserve vision after injecting into eye.^{6,16–19} The purpose of this study is to investigate whether hESC-derived NSCs induced by small molecules can preserve vision when injected into the Royal College of Surgeons (RCS) rat, a well-established retinal degeneration model. In the RCS rat, the retinal pigment epithelial cell (RPE) fails to phagocytose shed outer segment material due to a mutation in the MertK gene.^{20–22} This results in an accumulation of outer segment debris and subsequently leads to photoreceptor cell death. A comparable defect has been observed in a cohort of patients with RP.²³ The RCS Rat model also serves, to some degree, as a model for AMD since the photoreceptor

loss is due to defective or dysfunctional RPE cells. Preclinical studies of hES-RPE cells²⁴ were conducted in this animal and are currently in clinic trials for the treatment of AMD.

Methods

NSCs

Derivation of human NSCs from hESC was described previously.¹⁵ Briefly, hESC, H1 (passages 40–50) at 20% confluence were treated with 3 μ M CHIR99021 (Cellgentech, San Diego, CA) and 2 μ M SB431542, and 0.1 μ M Compound E (γ -Secretase Inhibitor XXI; EMD Chemicals Inc., Billerica, MA) in neural induction media containing Advanced Dulbecco's Modified Eagle's Medium (DMEM)/F12: Neurobasal media (1:1; Invitrogen, Carlsbad, CA), 1% Glutamax (Invitrogen, Carlsbad, CA), N2, B27, 10 ng/mL human leukemia inhibitory factor (hLIF), and 5 μ g/mL bovine serum albumin (BSA), supplemented with 3 μ M CHIR99021 and 2 μ M SB431542 for 10 days. Subsequently, the induced NSCs are expanded and maintained in the same neural induction media.¹⁵

NSC-Green Fluorescent Protein

NSCs were labeled with enhanced green fluorescent protein (EGFP) by lentiviral transduction and stable integration into the genome. EGFP was under the control of human E2F-a promoter. NSCs were maintained in a chemically defined media containing advanced DMEM/F12:Neurobasal (1:1), 1 \times N2, 1 \times B27 (Invitrogen, Carsbad, CA), 1% Glutamax, 5 μ g/mL BSA, and 10 ng/mL hLIF (Millipore, Billerica, MA), supplemented with 3 μ M CHIR99021 and 2 μ M SB431542 on Matrigel-coated plates (BD Bioscience, San Jose, CA).

Experimental Groups

RCS rats at postnatal day (P) 22 were divided into the following groups: (1) received subretinal injection of NSCs ($n = 14$) and carrying medium, balanced salt solution (BSS) ($n = 10$), (2) received subretinal injection of NSC-GFP in BSS ($n = 10$), (3) received intravitreal injection of NSCs in BSS ($n = 10$) and BSS alone ($n = 10$), and (4) untreated RCS and wild type rats were used as untreated controls ($n = 12$).

Subretinal Injection

A cell suspension containing approximately 3×10^4 NSCs in 2 μ L BSS was injected into the subretinal

Table. Sources and Working Dilutions of Antibodies Used in This Study

| Antibody | Working Dilution | Source |
|--------------------------|------------------|---------------------------------|
| Bestrophen (m) | 1:100 | Millipore |
| Brn 3 (g) | 1:200 | Santa Cruz Biotechnologies |
| Calbindin (rab) | 1:1000 | Swant, Marly, Switzerland |
| CD11b (m) | 1:100 | Millipore |
| CNTF (rab) | 1:1000 | Abcam, Cambridge, MA |
| CtBP2 (m) | 1:300 | Santa Cruz Biotechnologies |
| Glutamine synthetase (m) | 1:1000 | Millipore |
| Human nuclear marker (m) | 1:300 | Millipore |
| Iba1(g) | 1:1000 | Abcam |
| Ki67 (rab) | 1:500 | Epitomics, Burlingame, CA |
| Nestin (rab) | 1:200 | Millipore |
| Pax 6 (rab) | 1:500 | Covance, Princeton, NJ |
| PKC α (rab) | 1:1000 | Santa Cruz Biotechnologies |
| PNA-Alexa 488 | 1:200 | Life Technologies, Carlsbad, CA |
| PSD-95 (m) | 1:1000 | Millipore |
| Recoverin (rab) | 1:1000 | Millipore |
| RPE 65 (m) | 1:300 | Millipore |

space through a small sclera incision with a fine glass pipette (internal diameter, 50–75 μ m) attached by tubing to a 25- μ L 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 the same, except BSS alone was injected.

Intravitreal Injection

A cell suspension containing approximately 3×10^4 NSCs in 2 μ L BSS was injected into the vitreal cavity via the ora serrata at an angle to avoid damaging the lens. The cornea was punctured before injection to limit the efflux of cells. A sham group was treated the same, except BSS alone was injected.

Immediately after injection, the fundus was examined to check for retinal damage or signs of vascular distress. Any animals showing such problems were removed from the study and are not included in the animal counts herein. All procedures were conducted on pigmented RCS rats, which received daily intraperitoneal injections of dexamethasone (1.6 mg/kg) for 2 weeks following transplantation to control acute inflammatory responses and cyclosporine A in drinking water (210 mg/L) throughout the experimental period.²⁵ These studies were conducted with the approval and under the supervision of the Institutional Animal Care Committee at the Oregon Health & Science University and adhered to the

ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Functional Evaluation

Optokinetic Response (OKR)

Visual acuity was tested on all the animals by OKR from P60 to P160 using an optomotor testing apparatus²⁶ with our published protocol.^{27–29}

Luminance Thresholds (LT)

To measure LTs, we recorded single and multiunit activity in the superficial layers of the superior colliculus (SC) from P90 to P180 by using a modification of a procedure we had developed in previous work.^{30,31} To monitor the efficacy of NSCs in preserving vision over time, individual animals were recorded three times (P90, P150, and P180) before being sacrificed for histology.

Histology

At the end of functional tests, rats were euthanized with CO₂. Eyes were removed and immersed in 2% paraformaldehyde for 1 hour. Eyes were infiltrated with sucrose and embedded in optimum cutting temperature compound (OCT). Horizontal frozen sections (10 μ m) were cut on a cryostat. Sections were stained with cresyl violet for assessment of the injection site and retinal lamination. Extra retinal sections were stained with antibodies according to our

previous protocols.³⁰ The antibodies used in this study are listed in the [Table](#). Retinal sections were examined by regular and confocal microscopy.

Results

NSCs Offered Preservation of Vision When Delivered to the Eye with Retinal Degeneration

RCS rats received unilateral injection of NSCs, NSC-GFP, or carrying medium alone at P22, the fellow eye was untreated as internal control for each animal. All animals were maintained under immunosuppression by adding cyclosporine A in drinking water (210 mg/L) through the experimental period. OKR was conducted at P60, P90, and P160 to examine spatial visual acuity. In the RCS rat, the visual acuity deteriorated as degeneration progress, from 0.52 ± 0.03 cycle/degree (c/d) at P30 to 0.31 ± 0.04 c/d at P90, and 0.03 ± 0.04 c/d at P150. Subretinal injection of NSCs significantly preserved visual acuity over controls ([Fig. 1A](#)). At P90, an average of 0.475 ± 0.04 c/d was recorded in cell-injected eyes compared with 0.31 ± 0.02 c/d in medium-injected, and 0.29 ± 0.01 c/d in untreated controls ($P < 0.0001$, one-way analysis of variance [ANOVA]). There is no significant difference between medium-injected and untreated eyes ($P > 0.05$). Even at P160, while visual acuity in the control animals had declined to 0.06 c/d, NSCs-injected animals still performed at 0.40 ± 0.02 c/d, which is about 77% of the value at P30, indicating NSCs preserved visual acuity for over 4 months post injection.

Next we asked how retinal functional deterioration is limited after subretinal injection of NSCs by recording luminance thresholds in the SC. The luminance threshold recording from the SC is a unique method to examine retinal functional sensitivity across the visual field, which in turn provides a geographic indication of the magnitude and area of preservation across the retina. The use of retinal sensitivity measurements provides a background for assessing higher visual functions in the rats and a direct comparison for human perimetry measures. In the RCS rats at P90, the threshold levels were greater than 3.0 log units above the background level of 0.02 cd/m². In the wild type rats, the threshold is less than 0.5 log units above background. In this study, rats were selected according to OKR results since the

recording is very time consuming, it is impossible to collect data within a limited time frame from all the animals. [Figure 1B](#) shows that NSC injected eyes had much lower LTs compared with controls ($P < 0.001$), indicating more sensitive to light stimulation in cell-injected eyes. Specifically, over 85% of the SC area of NSC-injected eyes gave thresholds lower than 2.06 log units, with the most sensitive point falling within the normal wild type range. In comparison, less than 5% of the SC area produced thresholds less than 2.06 log units in sham-treated eyes and 0% of the SC area in untreated eyes. [Figure 1C](#) showed LTs in an individual rat with cell-injected (right eye) and untreated fellow eye (left eye). It is noted that cell-injected side has much lower LTs than untreated control side. The cell-injected rats were subsequently recorded at P150 and P180. Thresholds across the range recorded were significantly better at P90 than at P180 ($P < 0.005$); more than 50% of the SC area gave thresholds lower than 1.47 log units at P90 compared with 20% of the SC area at P180. This indicates that NSC injected eyes sustain higher sensitivities to light stimulation over a long term; however, there is some deterioration with time. It should be noted that luminance results gave best case performances, as opposed to the visual acuity tests (OKR), in which all rats were measured.

Subretinal Injection of NSC-GFP

It has been a concern that transfection with GFP may affect cell function. In this study, we found that NSC-GFP injected eyes performed significantly better in visual acuity test by OKR than controls ($P < 0.001$). At P90 when medium and untreated eyes performed at 0.32 c/d, NSC-GFP-injected eyes gave a figure of 0.50 c/d; this difference was highly significant ($P < 0.001$) ([Fig. 1D](#)). At the same time point, visual acuity in nonlabeled NSC-injected eyes was 0.48 c/d. The difference between these two groups was not significant ($P > 0.05$) ([Fig. 1E](#)). This study revealed that NSC transfection with GFP did not affect efficacy of the cells in preserving visual function.

Intravitreal Injection of NSCs

To investigate whether NSCs can exert their effect at a distance from the photoreceptors rather than only through cell contact mediated events, NSCs were injected into the vitreous cavity. We found the cell-injected eyes performed significantly better on OKR test than medium-injected, and untreated controls ($P < 0.001$) ([Fig. 1F](#)), indicating

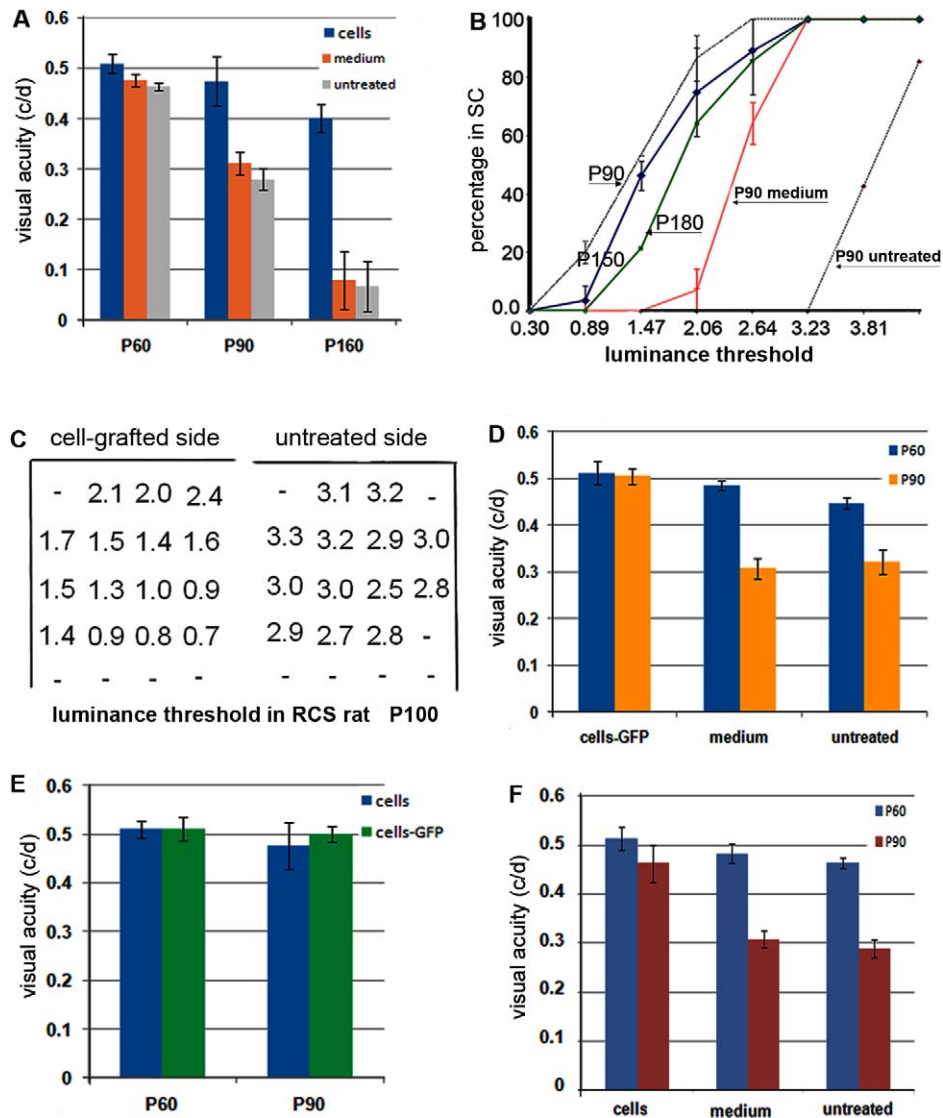


Figure 1. Functional evaluation of NSC, sham-injected, and untreated animals. (A) Visual acuity was tested by OKR at P60, P90, and P160 on animals received subretinal injection of NSCs, sham (BSS), and untreated as controls. Cell-injected eyes performed significantly better than controls at all the time points tested ($P < 0.001$). At P160, cell-injected animals still had visual acuity of 0.40 ± 0.02 c/d, which is about 77% of the value measured at P30, indicating NSCs preserved visual acuity for 4 months post injection, while control animals were almost non-responsive. (B) Luminance thresholds were recorded from the SC, which examines functional sensitivity across the visual field. Rats were recorded longitudinally for three time points (P90, P150, and P180) to study the efficacy of NSC grafting with time. It revealed that NSC injected eyes had significantly lower luminance thresholds compared with controls ($P < 0.001$). For examples, over 85%, 70%, and 60% of the SC area have luminance threshold lower than 2.06 log units at P90, P150, and P180, respectively, only 5% of the SC area has luminance thresholds lower than 2.06 log units in medium injected animals at P90. It is noted that there is deterioration over time even in NSC treated eyes. (C) Row data recorded from NSC-grafted and untreated sides in the SC at P100. It showed that luminance thresholds are much lower on the cell-grafted side than the untreated side. Typically in the RCS rat at P100, luminance thresholds are around 2.5 to 3.0 log units; some points on the cell-grafted side are over 100 times more sensitive to light stimulation than untreated control side (e.g., 0.7 vs. 2.9). (D) Visual acuity in animals received subretinal injection of NSCs labeled with EGFP by lentiviral transduction and stable integration into the genome (NSCs-GFP) was tested at P60 and P90 by OKR. It revealed that NSCs-GFP injected animals performed significantly better than sham and untreated controls ($P < 0.001$). (E) Visual acuity in animals received subretinal injections of NSCs and NSCs-GFP. Data from NSC and NSCs-GFP treated group was compared; it revealed that there was no statistically difference ($P > 0.05$) between the groups at both time points (P60 and P90) tested by OKR. (F) Efficacy after intravitreal injection of NSCs. NSCs were injected into vitreous cavity (same dose as subretinal injection); OKR was conducted at both P60 and P90. It showed that intravitreal injection of NSCs produced significantly better visual acuity than sham and untreated controls ($P < 0.001$), indicating trophic effect of NSCs in preserving vision.

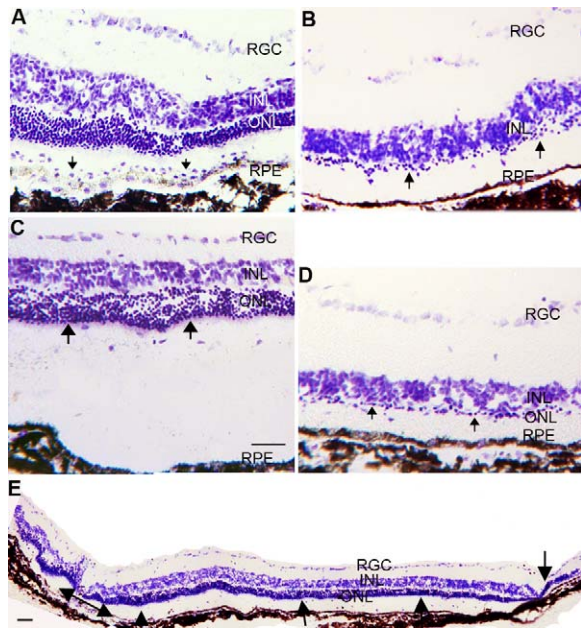


Figure 2. Retinal sections were stained with cresyl violet to examine photoreceptor preservation and general retinal lamination. (A) At P90, there are seven to eight layers of ONL in NSC grafted area (arrows indicated grafted cells), however only one layer of ONL was remaining in sham-injected retina (B). (C) Intravitreal injection of NSCs also produced robust ONL preservation (arrows), while in medium injected control (D), there was one layer of ONL remaining (arrows). (E) Retinal montage image of subretinal injection: it showed the preservation of photoreceptors (up pointing arrows), injection site (double arrows), and area with a single layer of ONL (down pointing arrow). Scale bars = 50 μ m.

the efficacy of NSCs was, at least partially, via trophic factors.

Transplanted NSCs Survived, Integrated, and Preserved Photoreceptors When Delivered to the Eye with Retinal Degeneration

Subretinal injection of NSCs significantly preserved photoreceptors compared with controls at all the time points examined (P60, P90, and P180). At P90 when both sham-injected and untreated eyes had a single layer of photoreceptor cell bodies in the outer nuclear layer (ONL), NSC treated eyes still had eight cell layers in the ONL (Fig. 2A versus 2B). Even at P180, there were still four to five layers of photoreceptors in NSC injected eyes. Retinal lamination was also maintained in graft protected areas; while in controls, there were only sparsely distributed photoreceptor cell bodies, and the normal retinal lamination was disrupted by secondary vascular pathology as reported previously.³²

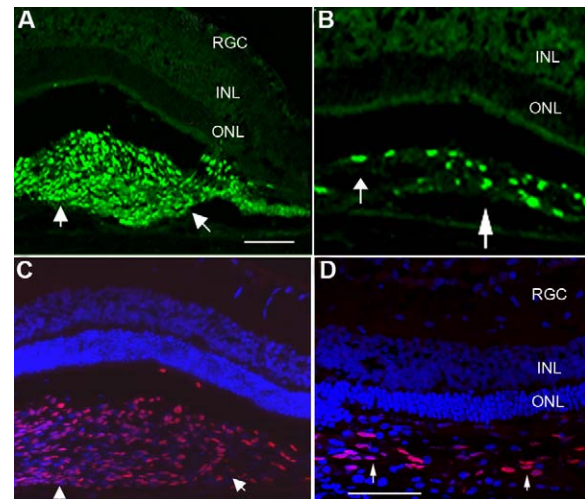


Figure 3. Confocal images showing the distribution of NSCs-GFP and NSCs (revealed by antibody MAB 1281, red) after subretinal injection. At the early time point after injection (P45, just over 3 weeks post injection), donor cells formed lump of cells after subretinal injections of both NSCs-GFP (arrows in [A]) and NSCs (arrows in [C]); donor cells formed a layer of two to three cells thick in the subretinal space at P90 (arrows in [B] and [D]). Sections were counterstained with DAPI (blue). Scale bars = 50 μ m.

Intravitreal injection of NSC also produced substantial photoreceptor preservation, with 8 to 10 layers of cells in the ONL in NSC treated eyes at P90, compared with two layers in sham-injected eyes (Fig. 2C versus 2D). NSCs were found in the vitreal cavity, along lens capsule, adjacent to retinal ganglion cell layer, not in other area such as trabecular meshwork and ciliary body. Subretinal injection of NSC-GFP also significantly protected photoreceptors from degeneration and preserved normal retinal lamination. The distribution of NSC-GFP changed from a lump of cells at 2 weeks post injection (Fig. 3A), spread in the subretinal space over time and eventually formed a layer of one to two cells thickness at P90 (Fig. 3B).

To identify the distribution of grafted cells, human specific nuclear marker (MAB1281) was used as before.^{6,30} Like the NSC-GFP grafts, antibody positive cells were seen as a lump of cells at 2 weeks post grafting (Fig. 3C), and a layer of one to two cells thick in the subretinal space at P90 and beyond (Figs. 3D, 4A, 4C). The subretinal grafted NSCs were found at two distinct locations: one as a one to two cells thick layer in the subretinal space, and another as individual cells distributed within the inner retina (Figs. 4A, 4C). The NSCs in the subretinal space contained intracellular pigment granules similar to host RPE cells; while the individually distributed cells

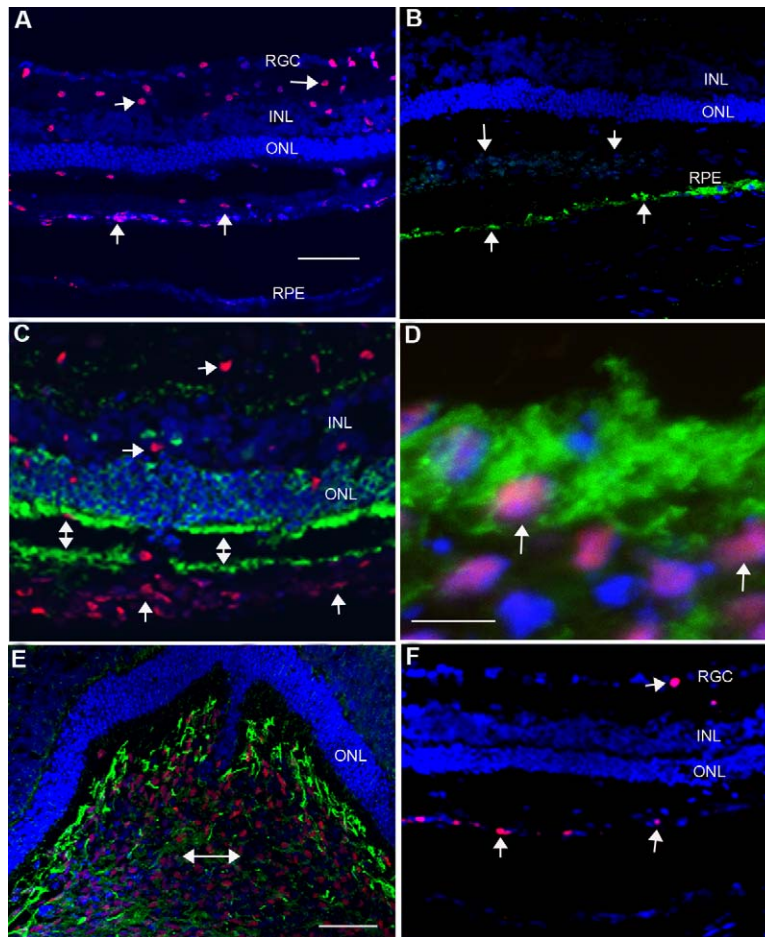


Figure 4. NSCs had two distinct locations: one formed a layer in the subretinal space (*up pointing arrows* in [A]); another one dispersed in the inner retina (*right pointing arrows* in [A]). There was robust photoreceptor preservation even at P180 (A). (B) Retinal section next to (A) stained with antibody RPE65 showed host RPE cells were positively stained (*up pointing arrows* in [B]), while donor cells were negative for RPE 65 (*down pointing arrows* in [B]). (C) Retinal section was double stained with human nuclear marker (*red*) and recoverin (*green*), there was no colocalization. (D) High power image showing donor cells (*purple*) were surrounded by recoverin positive materials (*green*). (E) Retinal section was double stained with human nuclear marker (*red*) and human nestin (*green*), it showed that donor cells expressed nestin. (F) Retinal section was stained with human specific antibody to Ki67, it showed a few positive stains in the subretinal space (*up pointing arrows*) and inner retina (*right pointing arrow*). Scale bars = 50 μm for (A) and (E), and 10 μm for (D).

in the inner retina did not contain pigment. By comparing the areas of ONL preservation and NSC distribution, we found that photoreceptor rescue was correlated with NSC location in the subretinal space (i.e., the thickness of the photoreceptor layer was gradually reduced outside the area of subretinal located donor cells). The photoreceptor layer was reduced to a sparsely distributed, single layer at P180 in areas distant from the subretinal graft, despite the presence of obvious NSCs within the inner retina in these regions. This suggests that NSCs located in the subretinal space were responsible for the photoreceptor preservation.

Next, we asked if these NSCs differentiated into mature cells in the retina, we applied two character-

istic RPE cell markers, RPE65 and bestrophin, to retinal sections containing NSCs in the subretinal space. There was no positive staining in the area where the grafted NSCs were distributed, while host RPE cells had strongly positive staining (Fig. 4B), which argued against that these NSCs differentiated into RPE cells. Further, we applied antibodies against Ibal and CD11b, which label microglia markers; we did observed activation of microglia in the retina, but there was no double labeling with MAB1281 on the grafted NSCs. These results suggest that the grafted NSCs did not differentiate into RPE cells or microglia. To examine whether NSCs migrated into the inner retina differentiated into retinal cells such as photoreceptors, bipolar cells, or retinal ganglion cells,

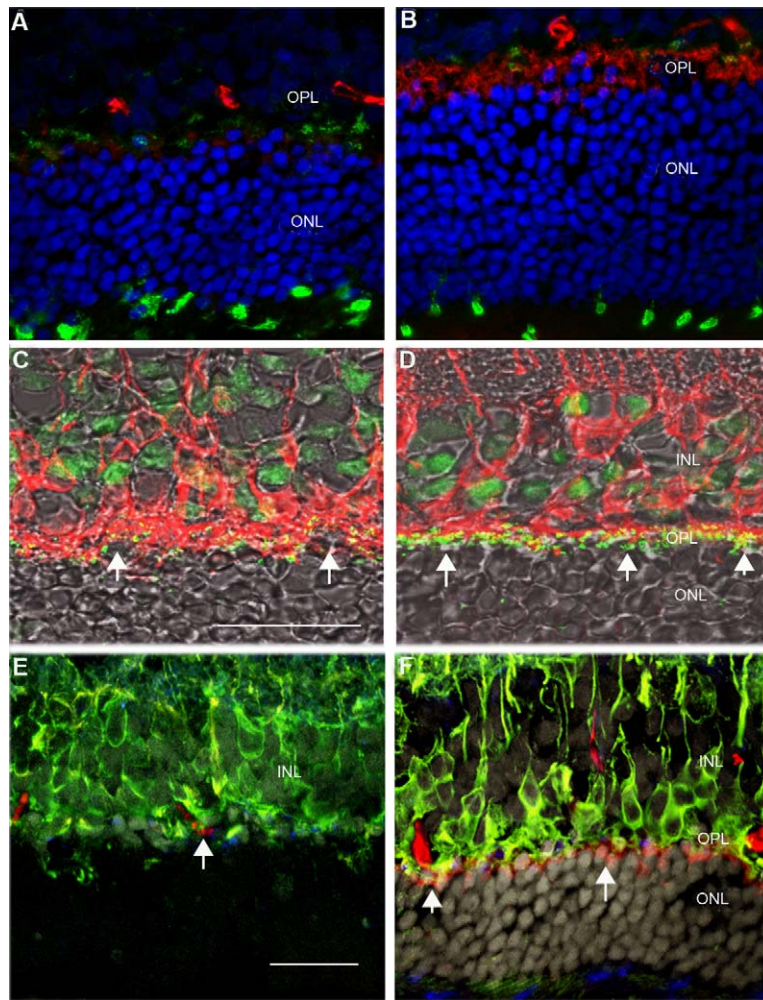


Figure 5. Confocal images showed NSCs preserved photoreceptors and limited secondary modifications: (A) and (B) Retinal sections from NSCs-grafted eye at P45 were double stained with PSD95 (red) and PNA-lectin (green), counterstained with DAPI (blue). It revealed that PSD95 was well preserved in graft-protected area (B) compared with area distance from the graft (A), however, cone outer segments (revealed by PNA-lectin) were similar in graft-protected area and distance from the grafts. (C) and (D) Showed organized distribution of C-terminal binding protein 2 (Ctbp2) staining (green, arrows in [D]) in graft-protected area, compared with area distance from the graft (arrows in [C]) at P45 and rod bipolar cells (red) were revealed by PKC α staining. (E) and (F) Rod bipolar cells (green) also underwent changes as degeneration progresses, there is hardly any dendrites left (arrow in [E]) in area away from the grafts, while in graft-protected area, the dendrites of bipolar were preserved and formed connection with PSD95 (red, arrows in [F]) even at P100. Scale bars = 20 μ m.

double labeling of grafted cells with antibodies against retinal neurons and antibody MAB 1281 was performed. Again, these migrated NSC failed to express any retinal neuronal markers examined (including recoverin [Figs. 4C, 4D], PKC α , calbindin, and brn-3 [data not shown]). This result indicated that NSCs migrated into inner retina did not differentiate into retinal neurons. To further examine whether the grafted NSCs still remain as neural stem cells and have the ability to proliferate, antibodies against NSCs such as nestin, Pax6, and Ki67 were used on retinal sections with NSC grafts. We observed that NSCs in subretinal space or the inner retina

distributed still expressed nestin (Fig. 4E), a neural stem and progenitor cell marker, but not Pax6. A small portion of NSCs were also positive for Ki67 (Fig. 4F), indicating continued cell division. We did not observe any tumor formation up to P180, the longest survival time in this study.

It is well documented that the secondary pathological changes occur as primary photoreceptor loss starts. To examine whether grafted NSCs also preserved the secondary retinal neurons and inner retinal connections, retinal sections (3 weeks post grafting) were stained with postsynaptic density protein 95 (PSD95) (red) and PNA-Alexa 488 (Life

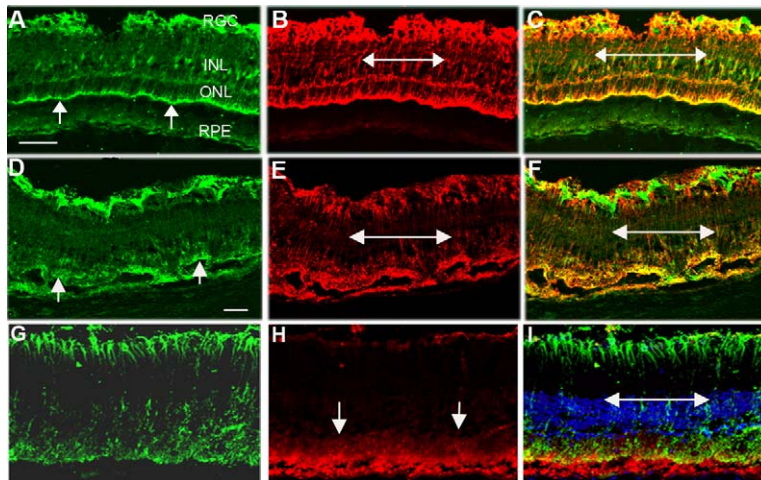


Figure 6. (A–C) Retinal section with subretinal injection of NSCs at P90 was double stained with glutamine synthetase (GS, green in [A]), CNTF (red in [B]), and (C) showed colocalization. (D–F) Retinal section with intravitreal injection of NSCs at P90 was double stained with GS (green in [D]) and CNTF (red in [E]), (F) showed colocalization. (G–I) Retinal section from age-matched, untreated RCS rat was double stained with GS (green in [G]) and CNTF (red in [H]), counterstained with DAPI (blue in [I]), there was almost no double staining in the retina (I). Scale bar = 50 μ m.

Technologies, Carlsbad, CA) (marker for cone outer segments and synaptic terminals, green) outside (Fig. 5A) or within (Fig. 5B) the graft-protected area. PSD95 staining was dramatically reduced outside the graft-protected area, while in the graft-protected area, several layers of labeled rod terminals were observed. PNA-labeled cone terminals were similar both beneath the graft and away from the graft, indicating cone structure is maintained at the early stage of degeneration. The active zones of rod and cone terminals were labeled with an antibody against the synaptic ribbon protein, C-terminal binding protein 2 (CtBP2). Rod terminal each contains a single large, arc-shaped ribbon, while cone terminals contain clusters of smaller ribbons. In NSC grafted retinas, the CtBP2 positive staining formed an organized array beneath the graft (Fig. 5D), while in areas away from the graft, the CtBP2 staining was less dense and disorganized (Fig. 5C).

Rod bipolar cells (rod-BC) are the unique output cells of rod photoreceptors. As rods are lost in the RCS retina, rod-BCs also undergo modifications. At P90 in NSC-injected eyes, the morphology of rod-BCs in the graft-protected area was fairly normal; their dendrites apposed rod synaptic ribbons (Fig. 5F) and contacted PSD95-labeled rod terminals. Away from the graft-protected area, rod-BC dendrites were reduced in length or completely lost, and their axons were also shorter (Fig. 5E). Sites of apposition between rod-BC dendrites and synaptic ribbons were sparser and more disorganized away from the graft-

protected area, and PSD95 labeling of rod terminals was absent or faint (Fig. 5E).

The mechanism of action of stem cell therapy is still not entirely clear. Neurotrophic factors, such as ciliary neurotrophic factor (CNTF) have been shown to be effective to retard progression of neurodegenerative disease including retinal degeneration in animal models^{33–36} and human clinical trials.³⁷ Previous studies shown that there was increased expression of CNTF in the retinal Müller cells after injection of bone marrow derived mesenchymal stem cells into the degenerated retina.²⁸ To investigate whether CNTF plays a role in this study, retinal sections were double-stained with antibodies against CNTF and glutamine synthetase (GS) (Müller glia marker). It was found that Müller glia cells increased expression of CNTF in NSC protected retina (Figs. 6A–C) in subretinal injection and in intravitreal injection (Figs. 6D–F). While in control (Figs. 6G–I) retinas, there was hardly any positive staining for CNTF. These data suggest that the grafted NSCs increased expression of CNTF by Müller cells in the graft-protected area.

Discussion

This study revealed that neural stem cells derived from hESC by small molecules offer marked protection of photoreceptors and visual function in a well established rodent model for retinal degeneration. Using both visual acuity and luminance threshold

tests at P90 and P180, NSC grafted eyes showed preservation of visual functions to levels among the best reported in the RCS rat models.^{25,38–48} The functional retention correlated with preservation of photoreceptors and inner retinal connections. In contrast, sham injection only had short term effect; previous studies revealed that subretinal injection of fibroblasts did not show efficacy in preserving vision in the same rat models,⁷ which further indicated that the rescue effect after grafting NSCs in this study are the results of specific event.

Histological study demonstrates that NSCs survive, migrate into inner retina after subretinal injection. These cells have two different appearances: one forms a layer one to two cells thick spreading in the subretinal space and contains pigment granules; another distributes into inner retinal and does not contain pigment. Close examination indicates that photoreceptor preservation is associated with the distribution of NSCs in the subretinal space; while the function of these cells distributed in the inner retina remains unknown. These results are very similar to our previous study using human forebrain derived progenitors (hNPC^{ctx}) and stem cells derived from the human brain (HuCNS-SC).^{6,43} Both hNPC^{ctx} and HuCNS-SC were isolated from post mortem fetal cortical brain tissue and cultured as spherical aggregates (neurospheres), showed that subretinal injection of hNPC^{ctx} into the same rat model produced a very similar outcome in terms of photoreceptor and vision preservation. Donor cells distribution (in the subretinal space and inner retina) and long term survival were also similar. With other cell types in the same model, we have not seen such significant migration and pigment-containing donor cells.^{7,27,41,49} In agreement with other studies grafting neural progenitors into the brain and retina,^{50–53} the NSCs do not express characteristic RPE cell markers, bestrophine and RPE65, and retinal cell markers. Whether NSCs localized in the subretinal space took on some RPE function needs further study at the ultrastructural level and requires examination of evidence for phagocytosis in the donor NSCs.

One of the main concerns in using embryonic stem cells is the risk of tumor formation. Our study showed that a small fraction of NSCs continued to divide after injection into the subretinal space. Cell proliferation marker (Ki67) positive cells were observed both in the subretinal layer and within the retina. However, there was no evidence of untoward cell proliferation or tumor formation up to 160 days post grafting. Therefore, it seems to suggest that cell

division is somehow regulated. Longer survival in these animals and immunocomprised animals is needed to make sure there is no tumor formation after injecting NSCs.

The efficacy of NSCs in preserving vision was unaffected by GFP transfection. Donor cell distribution at short term (P45) and longer term (P90) was similar (Fig. 3) between nonlabeled and GFP transfected NSCs; both formed a lump of cells post injection, and then spread into one to two cells thick layer in the subretinal space. Functionally, there is no significant difference in visual acuity tested by OKR. This provides important information for tracking donor cell distribution and fate in vivo using GFP prelabeling.

Cytokine mediated neural protection in animal models of degeneration was reported two decades ago,^{54–56} and has been reliably reproduced in the retina of many species with retinal degenerative diseases and other neurodegenerative conditions. However, the major problem for clinical application is that cytokines are rapidly degraded, the effect is short lasting. Repeated administrations run a high risk of damaging ocular tissue and complications such as infection related to the procedure. Cell-based, sustained delivery is a rational approach. Indeed, studies of stem cells grafted into degenerative models have demonstrated sustained neuroprotection.^{6,7,28,41,49,57,58} Here, we show that NSCs derived from hESC by small molecules produced robust preservation of photoreceptors and visual function after delivery into either the subretinal space or vitreous cavity in a well established rodent model for retinal degeneration. The mechanism by which NSCs preserve vision is not clear. Photoreceptor protection extends beyond the immediate boundaries of the donor cell distribution, indicating trophic action of diffusible factors. Furthermore, intravitreal injection of NSCs also offers great protection of both photoreceptors and visual function, reinforcing the trophic effect. It is widely accepted that stem cells exert their effort by modifying host responses. Our microarray data of in vitro cultured NSCs indicate expression of several growth factors; however, we did not see expression of CNTF. This is not surprising, as CNTF can be induced in an in vivo environment, which is drastically different compared with in vitro cell culture system. We found that host Müller glia upregulated expression of CNTF, which is well known for its neural protective effect. Currently, the mechanism of action of stem cells in preserving vision is still not fully understood. How do stem cells trigger host cells to release trophic factors, which in

turn exert its protective role? How is the gene expression changed before and after injecting stem cells into the eye? These are some of the critical questions needed to be addressed to understand how stem cells preserve vision.

In summary, neural stem cells derived from hESC by small molecules offer robust protection of photoreceptors and visual function. The major advantage of creating specific cell type by small molecules is that the cells can be generated more efficiently and stably maintained their characteristics over numerous passages without requiring integration of the virus to exert its effect. The grafted NSCs survived and integrated into degenerative retina, modified host responses. These NSCs offer great potential as a valuable cell source for treating degenerative retinal diseases.

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