Original Article

Mouse Degenerating Optic Axons Survived by Human Embryonic Stem Cell-Derived Neural Progenitor Cells

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Abstract

Objective: Any damage to the optic nerve can potentially lead to degeneration of non-regenerating axons and ultimately death of retinal ganglion cells (RGCs) that in most cases, are not curable by surgery or medication. Neuroprotective functions of different types of stem cells in the nervous system have been evaluated in many studies investigating the effectiveness of these cells in various retinal disease models. Neural progenitor cells (NPCs) secrete an assortment of trophic factors that are vital to the protection of the visual system. We aimed to assess the therapeutic potentials of NPCs in an ONC mouse model.

Materials and Methods: In this experimental study, NPCs were produced using noggin and retinoic acid from human embryonic stem cells (hESCs). Fifty mice were divided into the following three groups: i. Intact, ii. Vehicle [optic nerve crush+Hank's balanced salt solution (HBSS)], and iii. Treatment (optic nerve crush+NPCs). The visual behavior of the mice was examined using the Visual Cliff test, and in terms of RGC numbers, they were assessed by Brn3a immunostaining and retrograde tracing using DiI injection.

Results: Intravenous injection of 50,000 NPCs through visual cliff did not produce any visual improvement. However, our data suggest that the RGCs protection was more than two-times in NPCs compared to the vehicle group as examined by Brn3a staining and retrograde tracing.

Conclusion: Our study indicated that intravenous injection of NPCs could protect RGCs probably mediated by trophic factors. Due to this ability and good manufacturing practices (GMP) grade production feasibility, NPCs may be used for optic nerve protection.

Keywords: Human Embryonic Stem Cells, Optic Nerve Injury, Visual Cliff

Introduction

Nearly 0.5-5 percent of vehicular accidents lead to optic nerve crush (ONC) injuries; serious damages that could lead to cell degradation and eventual vision loss, due to the limitations in retinal ganglion cells (RGCs) regeneration (1).

Currently available medical interventions involving administration of neuroprotective medications such as corticosteroids to reduce inflammation, or surgery to remove pressure, have yielded little therapeutic success (2). Therefore, a large number of injured individuals-mostly of young ages-suffer from blindness (3). Nevertheless, it is anticipated that stem cells, which have the potential to cure neurological disorders, may help in overcoming this issue (4).

The following therapeutic methods are currently employed for neuropathological conditions: protecting the damaged cells, preventing further degeneration, and replacing the degenerated cells with cell transplants. RGC axons transfer the signals induced by visual stimuli in the eye to the brain's targets. Since RGC axons are very long and possess complex pathways, it does not seem logical to replace the degenerated cells with cell transplants. However, protecting the degenerating RGCs might be a promising approach.

Due to the protective and regenerative properties of stem cells, various types of these cells, including adult, embryonic and induced pluripotent stem cells at different levels of differentiation, have been studied in a variety of retinal disease models (5).

NPCs are located in the adult brain or derivatives from pluripotent stem cells. In the adult brain, they are found in two defined areas named subventricular zone (SVZ), which is around the ventricles of cerebral cortex, and subgranular zone (SGZ), located in the hippocampus. These parts of the brain are in charge of generating new neural cells. An injury or disease leading to neuronal loss and inflammation in the adult CNS will activate the NPCs by increasing their proliferation and migration rates. Studies have demonstrated that NPCs act mostly through

two main regenerative approaches: cell replacement and bystander effects (6).

In general, according to previous observations, conditioned medium properties and low integration of NPCs in retina, have led to the production of only a few regenerated axons from integrated cells. Application of NPCs is currently regarded as a more promising strategy for protecting the degenerating RGCs, due to their ability to secrete valuable neurotrophic factors (6).

Nonetheless, very few studies have examined the beneficial effects of NPC transplantation in the context of RGCs and photoreceptor cell defects in different eye diseases (7). Notably, these studies shared the fact that NPCs could protect the remaining RGCs and photoreceptors. However, functional replacement seems to be rare particularly in the case of RGCs that have long-distance innervating axons compared to the short-distance targeting axons of photoreceptors.

In this study, we aimed to assess the therapeutic potentials of NPCs in an ONC mouse model. We induced differentiation of human embryonic stem cells (hESCs) into NPCs, and subsequently injected them into tail veins of the ONC mice, in which ONC was induced two days prior to the injections. The purpose of this experiment was to determine the effects of NPCs on optic nerve function and probable long-term protection by evaluating RGC survival. Potential improvements of the NPC-conditioned medium led to secretion of some trophic factors including CNTF, bFGF and IGF1 (6). In this study, we hypothesized that intravenous (IV) injection of hESC-NPCs compared to its conditioned medium can improve functional recovery in ONC mice by paracrine effects, more efficiently.

Materials and Methods

Culture of hESC and Neuronal differentiation

In this experimental study, the hESC (Royan H6 line, passage 20) colonies were expanded and passaged according to the report by Mollamohammadi et al. (8). To generate expandable NPCs, hESCs were maintained and differentiated under serum and feeder-free conditions. The hESCs were induced to generate NPCs in two steps (6). The adherent colony culture of hESCs was treated with Noggin (R&D, 1967-NG, 100 ng/ml, USA) for six days (1) and the treatment was followed in the same medium with an increased concentration (250 ng/ml) of Noggin along with retinoic acid (Sigma-Aldrich, R2625, USA) for an additional six days. After appearance of the rosette structures, to reduce the contamination by other cells, they were manually picked up under phase-contrast microscopy and re-plated on poly-l-ornithine (Sigma-Aldrich, P4707, USA)/laminin (Sigma-Aldrich, L2020, USA) at a 1:15 volume/volume concentration. These structures were plated in NPC expansion medium containing DMEM F12, Knock out serum replacement (KSR) 5%, basic fibroblast growth factor (bFGF, Royan Biotech, Iran, 100 ng/ml) and epidermal growth factor (EGF, Sigma-Aldrich, USA, E9644, 20 ng/ml). After one week, the outgrowing colony

like cells were dissociated into single cells by 0.008% trypsin in 2 mM EDTA solution (Invitrogen, USA, 25300) and transferred to poly-l-ornithine (1:6)/laminin (1:1000) coated plates containing fresh NPCs expansion medium. The neural progenitor cells were passaged every 5-7 days at a ratios of 1:2 or 1:3 and remained proliferative with a highly homogenous morphology. For spontaneous differentiation, hNPCs received half the volume medium changes every 4 days in the absence of growth factors for 30 days.

Immunostaining

Immunofluorescence analysis was done according to standard protocols. In brief, we started with sample fixation in 4% paraformaldehyde (PFA, Sigma-Aldrich, USA, P6148) for 20 minutes at room temperature (RT), then, permeabilization using 0.1% Triton X-100 for 10 minutes. The samples were then incubated in blocking solution (10% secondary antibodies host serum) for 1 hour at RT, followed by an overnight incubation with primary antibodies at 4°C. Next, the cells were washed in phosphate buffered saline (BSA) and incubated with secondary antibodies for 45 minutes in an incubator at 37°C temperature. Table S1 (See Supplementary Online Information at www.celljournal.org) lists the primary and secondary antibodies used in this work. As negative control we incubated the cells with secondary antibodies only after the permeabilization step. Nuclei were stained by incubating the samples in 4, 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, USA, D9542, 5 µg/ml) or propidium iodide (Abcam, UK, ab14083) in PBS for 3 minutes at RT. The analysis was done under a fluorescent microscope (Olympus, Japan, IX71).

RNA isolation and polymerase chain reaction

Determining hNPCs identity was done by relative gene expression analysis versus undifferentiated hESCs. For this purpose, total mRNA was isolated from NPCs at passage 10, and from undifferentiated hESCs in triplicates by RNase Plus Universal Mini Kit (Qiagen, Germany, 73404). RNA purity and concentration were assessed by a UV/Visible Spectrophotometer (WPA, Biowave II). Then, the first-strand of cDNA was synthesized by 2 µg of total RNA by the Revert Aid First-strand cDNA Synthesis Kit and random hexamer primer (Fermentase, USA, k1632) in 20 µl reaction mixture, according to the manufacturer's instructions. Quantitative real-time RT-PCR was done in 20 µl PCR reaction containing 12.5 ng of synthesized cDNA in 2 µl and 10 µl 2x Power SYBR Green Master Mix (Applied Biosystems, USA) and 1 µl of 5 pmole forward and reverse primers. Reactions were run in a Rotor-Gene 6000 (Corbett Life Science, Australia). All qRT-PCR experiments were performed using three technical and three independent biological replicates. The amount of mRNA was normalized against *GAPDH* mRNA and compared using the ΔΔCt method. Primer sequences are presented in Table S2 (See Supplementary Online Information at www.celljournal.org).

Induction of optic nerve crush in mice

Male mice (C57BL/J6), at around 8-10 weeks of age, were kept on a 12 hours day/night cycle with free access to food and water. All animal trials were done in compliance with institutional guidelines and the ARVO statement for the use of animals in ophthalmic and vision research and Royan Institute ethic committee (IR.ACECR.ROYAN. REC.1397.251). The mice were anesthetized using a 1:4 mixture of xylazine/ketamine intraperitoneally (i.p). ONC was induced using fine forceps (tweezers #5B forceps, World Precision Instruments) according to the protocol in our pervious study (9). In summary, using an operating microscope (Olympus, Japan) left optic nerve (for behavioral test group left and right) was grasped approximately 1 mm from the globe for 5 seconds. Antibiotic ointments mixed of Gentamicin (Daroupakhsh, Iran) and tetracycline (Daroupakhsh, Iran) were administered for post-operative infection control. After ONC, the animals were randomly divided into the vehicle or hESC-NPCs groups.

Cell transplantation

C57BL/J6 mice were divided in defined groups as follows: i. Intact, ii. Vehicle, and iii. NPCs group. Unlike the vehicle and the hESC-NPCs groups, no injuries were made in the intact group (i.e., healthy mice), which comprised of mice of the same age as the ones in the other two groups. For determining the protective effects of intravenous injection of hESC-NPCs on the crushed nerve of the mice, the animals were held and fed under optimal conditions within 60 days from induction of the injury. On days 2, 4, and 6 after causing the injury, 200 µl of HBSS was injected to the tail vein of each mouse of the vehicle group (9) while the mice in the hESC-NPCs group received 50,000 cells in the same manner. We evaluated different doses of 100,000 and 50,000 cells and observed a higher survival rate after IV injection of 50,000 NPCs (data not shown). On day 60 after injury induction, the animals underwent behavioral tests and then, the murine retinas were isolated and subjected to various tests in order to determine the protective effects of the injected cells.

Visual behavioral test

The visual cliff test was used to analyze depth perception and the fear of crossing the deep side of the platform in mice; this method shows the relationship between the eye and the visual cortex. The mice individually underwent the test in a box while being recorded on video for 120 seconds. The videos were then analyzed by two condition-blinded persons. The test was done for at least 8 mice in each group 60 days after the crush. The box was designed according to a previous study (10). To begin the test, the mice first entered the shallow area, and then the time spent to cross the border to the deep area was considered as the latency time. Also, the mean time spent on staying in shallow area was measured and compared among the groups.

Examination of retrograde tracing

Retrograde tracing was used to determine RGC axonal integrity rate after the crush and treatment. In this experiment we had four mice in each group. For this purpose, according to the Paxinos atlas, on a stereotaxic device, a hole was made in each mouse skull over the superior colliculus (SC), and 2 µl of 2% DiI was injected into each SC. After 7 days, the animals were euthanized and their retinas were extracted after perfusion with saline and 4% PFA. The retina was then placed on a microscope slide and photographed under an IX51 Olympus fluorescence microscope.

Comparing neuron survival between groups

After 60 days of optic nerve crush, at least 6 mice from each group were sacrificed and the eyes were fixed in 4% PFA overnight. Then, the cornea and lens were cut out and the retinas were separated to perform immunostaining to detect the transcription factor Brn3a, which is a marker of RGC nuclei in the eye. Twelve images were taken of six retinas from each retina quadrants. The photos were then examined manually and the number of RGCs was compared among the groups.

Statistical analysis

GraphPad Prism (version 8, USA) was used to test the differences in behavioral and whole mount tests. Values plotted in visual test and the whole mount data are presented as mean ± SD. ***P<0.001; One way ANOVA and the Tukey's post hoc tests were used for more confirmation.

Results

Generation and characterization of hESC- NPCs

Generation of NPCs from hESC and related cell morphologies are detailed in Figure 1A. We selected increasing Noggin concentrations during the first 2 weeks of differentiation by adding RA during days 7-12 of the study Around day 12, hESC-NPCs showed typical morphology of defined clusters as columnar cells with rosette structures. The rosette structures were detectable under phase-contrast microscope. Afterward, they were manually picked up and re-plated (considered passage 0) on poly-L-ornithin/laminin-coated plates for expansion. Fortunately, NPCs were passaged every 5-7 days and they had uniform spindle-like morphology (Fig.1A, cell morphology at passage 15).

NPCs were characterized for cellular and molecular key markers at passage 10-15 and were used in the current study. The NPCs expressed neural progenitor markers including NESTIN, PAX6, OTX2, N-Cadherin, SOX1 and SOX 2 at both gene and protein levels (Fig.1B, C), which confirmed their differentiation potency toward various neural cell subtypes. Moreover, upregulation of *OTX2* gene against *OLIG2* and *HOXB2* confirmed our NPC population rostral identity and their potency

to differentiate toward retinal lineage. Finally, our spontaneous differentiation analysis confirmed their neuronal (TUJ1, MAP2 and NF) and glial (GFAP) differentiating potencies (Fig.1D).
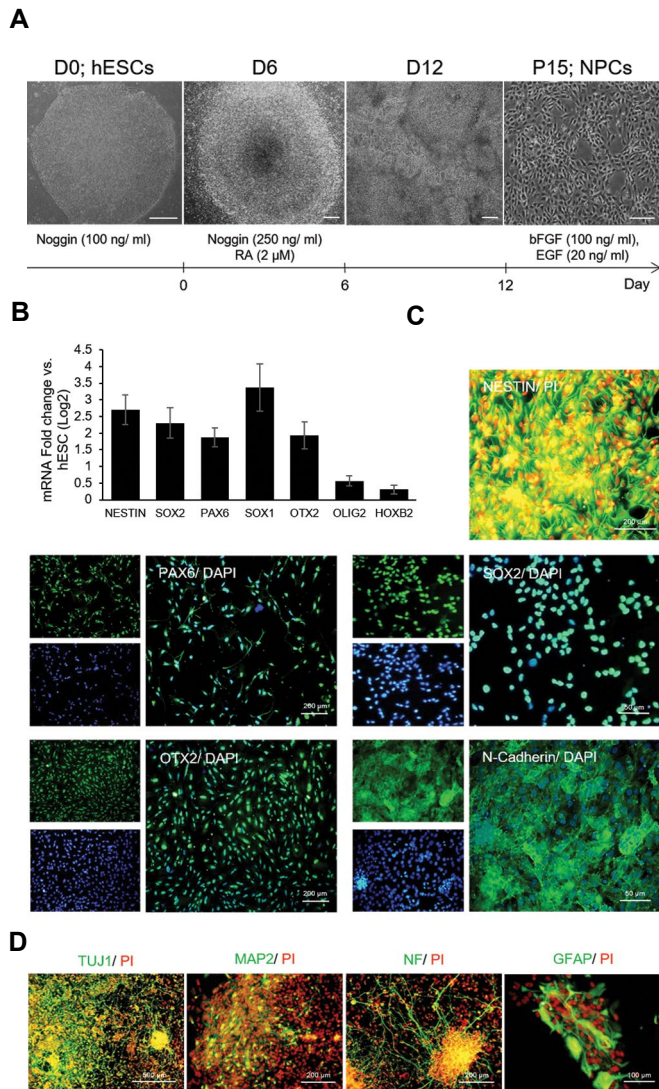
Fig.1: Characterization of hESC-NPCs. **A.** Timeline and phase-contrast images of the differentiation protocol used for generating NPCs from hESCs (scale bar: 200 µm). **B.** NPCs gene expression as assessed by qPCR. **C.** Fluorescent microscopic images of hESC-NPCs at passage 10-15 after immunostaining for NESTIN, N-Cadherin, PAX6, OTX2 and SOX2 as neural progenitor markers. Nuclei were counterstained with DAPI (blue) or PI (red). **D.** Fluorescent microscopic images of NPCs immunostaining after 30 days of spontaneous differentiation for TUJ1, MAP2, and NF as mature neural and GFAP as glial markers. Nuclei were counterstained with PI (red). hESCs; Human embryonic stem cells, NPCs; Neural progenitor cells, qPCR; Quantitative polymerase chain reaction, and PI; Propidium iodide.

NPC intravenous injection does not improve animal visual behavior

Figure 2A shows a schematic timeline of the present work. We injected 50,000 NPCs/200 µl HBSS intravenously via tail vein, 2, 4, and 6 days after the crush.

Mice were evaluated by visual cliff test for optic nerve regeneration. In this test, the time that was spent by the mice to cross the border between the shallow (safe area) and the deep (latency time) ends, was measured during two-minute periods. Considering the animals' fear of heights, latency time for the mice with healthy vision was longer. Our data showed that on day 60 post-injury, the average latency time for intact, vehicle and NPCs groups were 33.0 ± 6.13, 7.8 ± 3.5 and 14.5 ± 4.5 s, respectively (Fig.2B).

Total time in the safe area had the same trend in all groups, but the mice in the intact group stayed for a longer period of time in the shallow area compared to the vehicle and NPCs groups. Visual Cliff data were analyzed by one way ANOVA and showed no significant increase in latency time and time spent in the shallow area in animals that had received NPCs compared to the vehicle group, after two months.
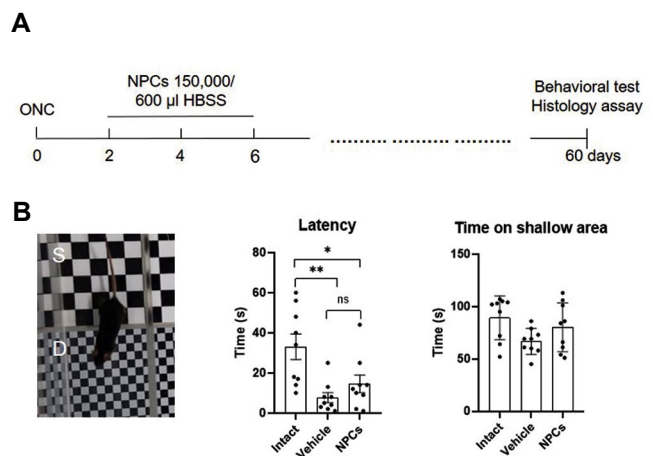

Fig.2: Study timeline and behavioral test. **A.** Schematic timeline of the intravenous injection of cells or HBSS (optic nerve crush time was considered day 0). **B.** Visual behavioral test was done 60 days after induction of the crush. Mouse in the visual cliff box (view from the top) with passing of the border between shallow (S) and deep area (D), Visual behavioral test was done on day 60 with non-significant difference of decision time between NPCs and Vehicle group, n=9 for each group. Values plotted are mean ± SD; unpaired t test. HBSS; Hanks' balanced salt solution and NPCs; Neural progenitor cells.

NPCs improved neuroprotection in the retina

Compared to the vehicle groups, significantly higher RGC nuclei concentrations were found in the NPCs group as shown by immunofluorescent staining. After extracting the whole retinas, the RGCs were detected by labeling the transcription factor Brn3a, which is a marker specifically used to stain RGC nuclei (Fig.3A). Average cell count of the whole retinas on day 60 per each group was as follows: Intact group: 688.68; Vehicle group: 158.66; and NPCs group: 404.74. Data were analyzed by GraphPad Prism using one-way ANOVA and Tukey's multiple comparisons test. Based on data obtained from counting the nuclei stained with Brn3a, the NPCs group showed a significant improvement compared to the vehicle group ($P<0.0001$, Fig.3B).

A

B
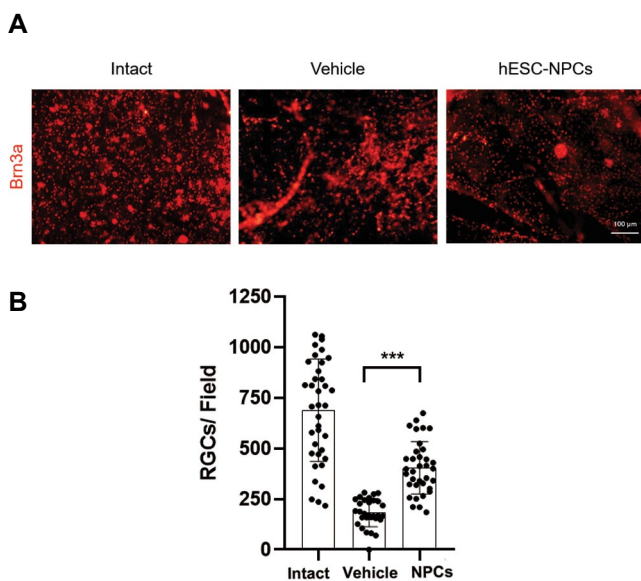
Fig.3: Survival rate in hESC-NPCs group compared to the vehicle and intact on day 60 post crush. **A.** Whole mount retinas stained with Brn3a against RGCs nuclei (scale bar: 100 μm). **B.** Average numbers of viable RGCs counted in 30-38 fields of four retinas from each of the intact, vehicle and NPCs groups. Data are presented as mean ± SD. hESCs; Human embryonic stem cells, NPCs; Neural progenitor cells, RGCs: Retinal ganglion cells, and ***; P<0.001, unpaired t test.

Retrograde tracing showed extensive neuroprotection

Retrograde tracing test was used to inspect the healthy RGC axons that deliver signals from the brain to the retina. For this purpose, DiI was injected in the SC of the mouse brain and was tracked in RGC bodies at 5 to 7 days post-injection. Photographs taken from at least four eyes in each group, were compared qualitatively. The results indicated a significant higher axon survival rate in the NPCs group compared to the vehicle group (Fig.4).
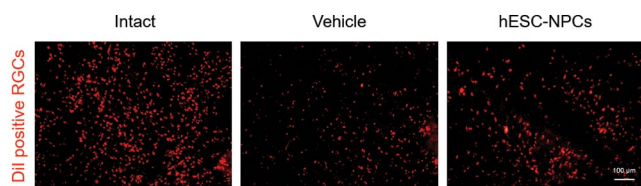
Fig.4: Retrograde tracing in the hESC-NPCs group compared to the vehicle and intact groups on day 60 after Crush. Retrograde tracing using DiI showed more RGC intact axons in the whole mount retinas of the NPCs-treated group compared to the vehicle group, n=4 for each group (scale bar: 100 μm). hESCs; Human embryonic stem cells, NPCs; Neural progenitor cells, and RGCs: Retinal ganglion cells.

Discussion

Optic nerve damage, caused by vehicular accidents or diseases, leads to degeneration RGCs, which have a very limited regeneration rate in mammals. This will ultimately cause permanent impaired vision or even blindness. Anti-inflammatory medicines such as corticosteroids as well as surgical interventions have been proven effective in delaying disease progression and removing the pressure from the nerve, but these solutions have limited outcomes. Thus, researches have been looking for novel approaches like using different stem cells.

Over the recent years, stem cells have created new hope for curing neurodegenerative diseases. Due to their protective and regenerative potentials, various types of stem cells such as the NPCs are being used; NPCs are adult stem cells in the central nervous system that can nowadays be derived from pluripotent stem cells.

Studies have shown that transplanted NPCs are able to migrate to the injury site, and after homing, they can apply this bystander effect through multiple displays including secretion of neurotrophic cytokine (e.g. NGF, VEGF, GDNF, NT-3, BDNF, etc.) that are vital for neural protection and can stimulate endogenous repair potentials of the residing progenitors (11, 12).

However, in the case of the trauma-induced neurodegenerative conditions occurring near a deleterious inflammatory environment, such as spinal cord or optic nerve crush injuries, or conditions that are due to a combination of genetic and environmental factors [e.g. Alzheimer's (13), Huntington's (14), and ischemic brain injury (15)], a simple replacement of the lost cells does not seem to be enough. Indeed, a potentially successful approach to treat such conditions should provide a multidimensional cross-talk between immune cells, neural progenitors and damaged mature neurons. Therefore, NPCs via exerting bystander effects, are still retained as a fascinating choice for cell transplantation in CNS diseases. In addition, it was demonstrated that NPCs can exert immunological properties by expressing various surface molecules, such as TLRs, chemokine receptors, integrins and specific cell adhesion molecules (11). However, the underlying cellular and molecular mechanisms are not completely understood at this time. Nonetheless, the results of clinical and preclinical studies on NPC transplantation in different neurodegenerative diseases (16) indicates that direct integration and replacement of the transplanted cells have insignificant (or even zero) impact on observed functional recovery, thus making the bystander effect hypothesis stronger regarding the NPC transplantation approach.

According to previous studies, after direct intravitreal injection of neurotrophic factors such as Ciliary neurotrophic factor, NT3 and VEGF, prevention of cell degeneration after the injury was observed in damaged RGCs (17). Based on the literature, trophic factors help RGCs to survive, but their use is limited due to high costs and the invasive nature of repeated injections. Secreted neurotrophic factors such as PDGF and BDNF are important for RGC protection (18, 19). NPCs secrete neurotrophic factors that improve the lesion microenvironment, thereby providing an appropriate condition for the repair (20).

Numerous studies have confirmed the positive effects of NPCs on regeneration of peripheral and central

nervous tissues (13). Also, NPCs have been proven effective in protecting neurons and the neural tissue in neurodegenerative diseases such as Parkinson's, stroke and spinal cord injury. Although the complete mechanism of this effect remains unknown, it is assumed to be a secondary event, which is dependent on neurotrophic factors such as IGF, NGF, CNTF, BDNF and FGF2 (12).

To date, only a few studies have investigated the potential effects of human pluripotent stem cell-derived NPCs on optic nerve regeneration. Banin et al., by subretinal or intravitreal transplantation of hESC-neural precursors in rat eyes, successfully showed the potential of these cells for retinal differentiation (21). Underlying mechanisms of cell therapy in the retina are still unclear. In addition, considering the complexity of retinal structure, we hypothesized that RGC regeneration is possible in the presence of NPC trophic factors. Therefore, continuous secretion of trophic factors by the NPCs injected systemically was considered for this study. Here, we showed that IV injected hESC-derived NPCs were beneficial for RGC survival without a loss of efficacy. Some studies on neural stem cell transplantation in neurological diseases, similarly suggested the function of neurotrophic factors as an underlying mechanism for neural regeneration (22, 23).

Our study showed that hESCs efficiently differentiated into neural progenitors using the Noggin protein as BMP- antagonist, and retinoic acid (RA) as a morphogen. The hNPCs expressed SOX1 and 2, NESTIN, PAX6, OTX2, and N-cadherin, and showed neural subtype differentiation potencies *in vitro*. Furthermore, NPCs had high expression levels of PAX6 and OTX2 markers of anterior brain and retinal differentiating linage cells (24, 25). According to our previous study, lower passages of NPCs derived from pluripotent stem cells, could express transcription factors that mostly confirmed the forebrain and rostral identity (26).

Since our NPCs had a rostral identity, they seemed to be suitable therapeutic candidates for degenerated RGCs. Moreover, we could claim that due to our NPC line homogeny along with less commitments toward a specific neural cell types, they have the capacity for homing properly, integrating in the injured optic nerve and releasing appropriate neurotrophic factors *in vivo* (27). Subsequently, they could change the inflammatory site toward noninvasive environment (in the site of injury), which will cause sufficient improvement as observed in the current study.

In the present animal study, C57 mice were used to provide an optic nerve damage model and on days 2, 4 and 6 each mouse received 50,000 ESC-derived NPCs over a 6-day period. We selected this cell therapy regimen since trophic factors are gradually secreted by NPCs. For selecting the hESC-NPCs dosage, our pilot study showed that triple IV injections of 50,000 cells is safe and appropriate.

To do this, at least 8 animals were tested in each group and the results, which were indicative of a cognitive behavior (i.e., the animal's fear of height), showed that different crossing times in the NPCs and vehicle groups carried no significant relationship; thus, we concluded that no behavioral improvement was achieved.

We found that NPCs significantly increased the survivability of RGCs compared to the vehicle controls. The significant neuroprotection offered by hESC-NPCs was confirmed by retrograde tracing test. This was performed through the injection of DiI into the SC in the brain. DiI entered RGCs and moved towards the cell body through the axons (28). Our results confirmed higher concentrations of DiI-stained RGCs in the NPCs group compared to the vehicle group.

Conclusion

These findings created new potentials for treating optic nerve damage using ESCs-derived NPCs. Further investigations should be carried out to help find a proper treatment for optic nerve damage. Taken together, human ES-NPCs promoted neuroprotection of RGC in ONC mice. The ease of transplantation without any side effects makes hES-NPCs an acceptable therapy for RGCs degeneration. Clearly, in translating these findings to the clinical applications, factors such as cell dosage and immune-related issues remain to be unraveled.

Acknowledgments

Authors' Contributions

Sh.N.; Data collection, analysis, interpretation, and manuscript writing. Z.S., S.S., M.H.; Collection and/ or assembly of data, data analysis and interpretation, and manuscript writing. S.K., F.P.; Data analysis and interpretation, and manuscript writing. M.J., L.S.; Conducting experiments, conception and design, data analysis and interpretation, administrative and financial support. L.S.; Manuscript proof. All authors read and approved the final manuscript.

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