

Induced Pluripotent Stem Cells Without *c-Myc* Ameliorate Retinal Oxidative Damage via Paracrine Effects and Reduced Oxidative Stress in Rats

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Abstract

Purpose: To investigate the efficacy and mechanisms of non-*c-Myc* induced pluripotent stem cell (iPSC) transplantation in a rat model of retinal oxidative damage.

Methods: Paraquat was intravitreally injected into Sprague–Dawley rats. After non-*c-Myc* iPSC transplantation, retinal function was evaluated by electroretinograms (ERGs). The generation of reactive oxygen species (ROS) was determined by lucigenin- and luminol-enhanced chemiluminescence. The expression of brain-derived neurotrophic factor, ciliary neurotrophic factor, basic fibroblast growth factor (bFGF), stromal cell-derived factor (SDF)-1 α , and CXCR4 was measured by immunohistochemistry and ELISA. An *in vitro* study using SH-SY5Y cells was performed to verify the protective effects of SDF-1 α .

Results: Transplantation of non-*c-Myc* iPSCs effectively promoted the recovery of the b-wave ratio in ERGs and significantly ameliorated retinal damage. Non-*c-Myc* iPSC transplantation decreased ROS production and increased the activities of superoxide dismutase and catalase, thereby reducing retinal oxidative damage and apoptotic cells. Moreover, non-*c-Myc* iPSC transplantation resulted in significant upregulation of SDF-1 α , followed by bFGF, accompanied by a significant improvement in the ERG. *In vitro* studies confirmed that treatment with SDF-1 α significantly reduced apoptosis in a dose-dependent manner in SH-SY5Y cells. Most transplanted cells remained in the subretinal space, with sparse cells expressing neurofilament M markers at day 28. Six months after transplantation, no tumor formation was seen in animals with non-*c-Myc* iPSC grafts.

Conclusions: We demonstrated the potential benefits of non-*c-Myc* iPSC transplantation for treating oxidative-damage-induced retinal diseases. SDF-1 α and bFGF play important roles in facilitating the amelioration of retinal oxidative damage after non-*c-Myc* iPSC transplantation.

Introduction

Oxidative stress has been implicated in many retinal diseases, including age-related macular degeneration and proliferative diabetic retinopathy.^{1–4} These diseases are the leading causes of blindness in developed countries. For most oxidative-stress-induced retinal diseases, the loss of retinal neurons is generally irreversible and results in blindness. Until recently, no effective treatment was available to repair damaged retinas in these diseases. Stem-cell-based therapies are considered novel strategies for treating incurable retinal diseases.

Induced pluripotent stem cells (iPSCs) have the potential for multilineage differentiation and can be a resource for stem-cell-based treatment. iPSCs can be induced from

somatic cells through reprogramming by transduction with defined transcription factors.^{5,6} iPSCs have been shown to be less immunorejective and lack the ethical concerns of embryonic stem cells.⁷ iPSCs can differentiate into various types of retinal cells,^{8,9} showing potential as replacement tissue for retinal diseases. However, this concept was challenged by recent observations that only small numbers of transplanted cells engraft into tissues. Increasing evidence has demonstrated that paracrine mechanisms, including anti-inflammatory activity and the release of neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), and basic fibroblast growth factor (bFGF), account for the therapeutic benefits of stem cells in experimental animal models.^{10–12}

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Stromal cell-derived factor (SDF)-1 α , a CXC chemokine, is a potent chemoattractant for leukocytes, endothelial progenitors, and hematopoietic stem cells.¹³ CXCR4 is the receptor for SDF-1, and the SDF-1-CXCR4 axis plays important roles in inflammation, tissue repair, and organogenesis.¹⁴ Several studies have demonstrated that mesenchymal stem cells can secrete SDF-1 α and promote the survival and regeneration of progenitor cells and cardiac myocytes.^{15–17} However, it remains unclear whether, after iPSC transplantation, SDF-1 α is involved in and regulates the recovery process after retinal oxidative damage.

Paraquat (PQ) is a bipyridyl herbicide capable of generating oxygen radicals. Cingolani et al. demonstrated that intravitreal injection of PQ induced diffuse oxidative damage to the retina in C57BL/6 mice. This oxidative damage resulted in apoptotic cell death, retinal morphologic changes, and reduced retinal function.¹⁸ In addition, intravitreal PQ injection is safe for local exposure of the retina, without systemic side effects. Therefore, intravitreal PQ injection is a good model for oxidative-damage-induced retinal diseases.

In this study, we attempted to reduce the incidence of teratoma formation after transplantation of iPSCs by using iPSCs without exogenous *c-Myc*. We evaluated the therapeutic effects of non-*c-Myc* iPSCs in a rat model of oxidative-damage-induced retinal diseases and explored possible mechanisms, specifically focusing on the roles of SDF-1 α . In addition, we evaluated the safety of transplanted non-*c-Myc* iPSCs by assaying for tumor formation 6 months after transplantation.

Methods

Reagents

PQ was obtained from Sigma-Aldrich. A DNA fragmentation detection kit (terminal deoxynucleotidyl transferase-mediated dUTP-biotinide end labeling [TUNEL]) was obtained from Calbiochem. The GFP antibody was purchased from BioVision. Mounting medium with DAPI and phycoerythrin streptavidin antibody were obtained from Vector Laboratories. Anti-nitrotyrosine was obtained from Abcam, anti-8-hydroxy-2'-deoxyguanosine was from JaICA, and anti-conjugated acrolein antibody was from Advanced Targeting Systems. Anti-CNTF and anti-bFGF antibodies were purchased from Millipore. Anti-BDNF and anti-CXCR4 antibodies were from Epitomics. Anti-mouse/rat SDF-1 α -purified antibody was from eBioscience. Mouse anti-neurofilament-M was from Invitrogen.

iPSC culture

We generated mouse iPSCs with only 3 exogenous genes (Oct4/Sox2/Klf4) and without the oncogene *c-Myc*. Murine iPSCs were generated from mouse embryonic fibroblasts derived from 13.5-day-old embryos of C57/B6 mice. The iPSCs were reprogrammed by transduction with retroviral vectors encoding 3 transcription factors, Oct-4, Sox2, and Klf4, as described previously.¹⁹ Undifferentiated iPSCs were routinely cultured and expanded on mitotically inactivated murine embryonic fibroblasts (MEFs; 50,000 cells/cm²) in 6-well culture plates (BD Technology) in the presence of 0.3% leukemia inhibitory factor in iPSC medium. Every 3–4 days, colonies were detached with 0.2% collagenase IV, dissociated into single cells with 0.025%

trypsin and 0.1% chicken serum in phosphate-buffered saline (PBS), and plated again onto MEFs. The non-*c-Myc* iPSCs were pluripotent and could be differentiated into 3 dermal-lineage cell types, including astroglial (neuroectodermal), osteogenic (mesodermal), and hepatocyte-like (endodermal) lineage cells, as described previously.^{19,20}

Animals and the retinal oxidative stress animal model

Sprague–Dawley (SD) rats weighing 150–200 g were used in all subsequent experiments. All animal experiments in this study adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

In an established animal model of retinal oxidative damage, 1 μ L of 0.5 mM PQ was injected into the vitreous cavity of rats.²¹ The rats were anesthetized with intraperitoneal 2% pentobarbital (40 mg/kg) and topical 1% proparacaine eye drops. Pupil dilation was achieved with 1% tropicamide. Sclerotomy was performed 1 mm behind the limbus with the tip of a 27-gauge needle. A 33-gauge blunt-tip needle (Hamilton) was inserted into the vitreous cavity, and 1 μ L of PQ was injected. The needle was left in the vitreous cavity for 1 min after injection to reduce the degree of reflux.

Animal grouping and subretinal cell transplantation

SD rats were randomly divided into 4 groups:

Group 1: Intravitreal injection of PBS, serving as a normal control (normal).

Group 2: Intravitreal injection of 0.5 mM PQ (PQ-injected group).

Group 3: Subretinal injection of MEFs 2 h after PQ injection (MEF-treated group).

Group 4: Subretinal injection of iPSCs 2 h after PQ injection (iPSC-treated group).

Total numbers of animals used in each group and the days to perform the experiments were summarized in Table 1. The treatment was blinded to the experimenter.

The right eye of each animal received injections. Rats were anesthetized, and pupils were dilated. The eyes were gently protruded using a rubber sleeve. A 90° peritomy was made in the temporal quadrant, and a sclerotomy was made with a 27-gauge needle. A 33-gauge blunt-tip needle was inserted tangentially toward the posterior pole of the eye, and 1 μ L of iPSC suspension ($\sim 1 \times 10^5$ cells/mL) or 1 μ L of MEF suspension was slowly injected into the subretinal space. Rats were sacrificed and the eyes were enucleated 3, 7, 14, 21, or 28 days after treatment.

Electroretinogram recordings

Rats were dark-adapted for 1 h before performing the electroretinograms (ERGs). All manipulations were done in dim red light illumination. After being anesthetized, the rats were placed on a heating pad, and a recording electrode was placed on the cornea after application of 0.5% methyl cellulose. A reference electrode was attached to the shaved skin of the head, and a ground electrode was clipped to the rat's tail. A single flash of light (duration = 100 ms) 30 cm from the eye was used as the light stimulus. Responses were amplified with a gain setting of $\pm 500 \mu$ V and filtered with low (0.3 Hz)– and high (500 Hz)–frequency cutoffs from an

TABLE 1. SUMMARY OF TOTAL NUMBER OF ANIMALS IN EACH GROUP PER EXPERIMENT AND DAYS TO PERFORM THE EXPERIMENTS

<i>Experiments</i>	<i>Number of animals in each group (N)</i>	<i>Days after treatment</i>
ERG	10	Days 3, 7, 14, 21, 28
Histology	3	Day 14
TUNEL and caspase III stain	3	Day 7
Immunofluorescence		
Nitrotyrosine, 8-OHdG, acrolein	3	Day 14
SDF-1 α , CXCR4	3	Day 14
bFGF, CNTF, BDNF	3	Day 14
CM-DiL	3	Days 14, 28
ELISA		
MDA, 8-OHdG	3	Day 14
SDF-1 α	3	Days 3, 7, 14, 21
bFGF, CNTF, BDNF	5	Days 3, 7, 14, 21
Chemiluminescence	3	Days 7, 14, 21
Antioxidative enzymes	3	Days 7, 14, 21
Western blot analysis- CXCR4	3	Day 7

BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; CNTF, ciliary neurotrophic factor; ERG, electroretinogram; SDF, stromal cell-derived factor; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotinide end labeling.

amplifier. The pattern of the a- and b-waves was recorded. The b-wave ratio was defined as the b-wave amplitude of the right eye divided by the b-wave amplitude of the left eye. The fold of the b-wave ratio represented the b-wave ratio at days 3, 7, 14, 21, or 28 divided by the b-wave ratio at day 0.

Histological study and retinal cell count

For every control and experimental animal, both eyes were collected 14 days after treatment. The specimens were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 30 min at 4°C and then washed with PBS. Five-micrometer sections were obtained using a cryostat, and some preparations were stained with hematoxylin and eosin (H&E) for morphological observations and retinal cell counts.

For retinal cell counts, the estimation of retinal rescue was made by comparing the total number of cells in the ganglion cell layer (GCL), the inner nuclear layer (INL), and the outer nuclear layer (ONL) of each eye. The total number of cells in the GCL, INL, and ONL were counted along ventral retinal sections from one margin to the other, indicated as areas 1 to 6, each 250 μ m in length along the section. The result was expressed as the cell number per 250 μ m of the retina.

Terminal deoxynucleotidyl transferase-mediated dUTP-biotinide end labeling

TUNEL assays were performed at day 7 using a DNA fragmentation detection kit according to the manufacturer's instructions. Sections were visualized on a fluorescence

microscope (Nikon Instruments). The mean number of TUNEL-positive retinal cells per 250 μ m length of retina section was counted.

Immunohistochemistry

Immunohistochemistry was carried out by simultaneously blocking and permeabilizing sections with 0.2% Triton in PBS containing 5% goat serum for 1 h at room temperature, incubating with primary antibodies diluted in blocking solution overnight at 4°C, and then incubating with the appropriate fluorescent secondary antibodies (all diluted 1:1,000) in blocking solution for 3 h at room temperature. Nuclei were counterstained with DAPI. Primary antibodies included GFP (1:5,000), caspase 3 (1:200), acrolein (1:200), nitrotyrosine (1:200), 8-OHdG (1:200), BDNF (1:200), CNTF (1:200), bFGF (1:200), stromal cell-derived factor (SDF)-1 α (1:200), and CXCR4 (1:100). Specimens stained with IgG isotype antibody as the primary antibody were used as negative controls. The mean number of caspase 3-positive retinal cells per 250 μ m length of retina section was counted.

The following formula was used for the densitometric quantitation of acrolein, nitrotyrosine, 8-OHdG, bFGF, CNTF, and BDNF immunohistochemistry, as previously described.²¹

Immunostaining index = $\Sigma [(X - \text{threshold}) \times \text{area (pixels)}] / \text{total cell number}$. Where X is the staining density indicated by a number between 0 and 256 in grayscale, and X is more than the threshold. Briefly, digitized color images were obtained as PICT files. PICT files were opened in grayscale mode using NIH image, ver. 1.61. Cell numbers were determined using the Analyze Particle command after setting a proper threshold.

The relative density of immunostaining was defined as immunostaining index of PQ-injected, MEF-treated, and iPSC-treated groups divided by immunostaining index of normal group.

Measurement of reactive oxygen species in the retina

Luminol and lucigenin chemiluminescence (CL) was measured as indicator of free-radical formation. Rats were sacrificed at days 7 and 14, and retinas were isolated and then homogenized in PBS. Lucigenin (bis-N-methylacridinium nitrate) and luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) were obtained from Sigma-Aldrich. Measurements were made at room temperature using a luminescence reader (Aloka Co.). Specimens were put into vials containing PBS-HEPES buffer. Reactive oxygen species (ROS) were quantified after the addition of enhancers, such as lucigenin or luminol, at a final concentration of 0.2 mM. Counts were obtained at 1-min intervals, and the results were given as the area under the curve for a counting period of 5 min. Counts were corrected for wet tissue weight (rlu/mg tissue).

Measurements of the activities of antioxidant enzymes in the retina

The levels of superoxide dismutase (SOD) and catalase activities in the retina were determined by colorimetric assays at days 7, 14, and 21. Both SOD and catalase assay kits were purchased from Cayman Chemical. Assay procedures

and tissue homogenate preparations were performed following the manufacturer's instructions.

Measurement of oxidative damage in the retina

The levels of MDA and 8-OHdG in the retina were measured at day 14 using MDA and 8-OHdG ELISA kits (Cayman Chemical and Cosmo Bio. Co.). The procedure was as described in the protocol provided by the manufacturer. The absorbance was measured at 450 nm using a microplate reader (Bio-Rad).

Measurement of the expression of bFGF, CNTF, BDNF, and SDF-1 α in the retina

The retinas of each group were harvested at days 3, 7, 14, and 21. These retinas were homogenized in 1 mL of PBS containing antiproteases (0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzethonium chloride, 10 mM EDTA, and 20 kallikrein inhibitor unit aprotinin A) and 0.05% Tween 20. The samples were then centrifuged at 3,000 *g* for 10 min, and the supernatants were immediately assayed using the bFGF and CNTF and BDNF ELISA kit (Cusabio), and SDF-1 α ELISA kit (Eagle Bioscience).

Western blot analysis

The rats in each group were sacrificed, and retinas were isolated at day 7. Total protein was extracted from the retinas by lysing the sample in radioimmunoprecipitation assay buffer. The extract and Laemmli buffer were mixed in a 1:1 ratio, and the mixture was boiled for 5 min. Samples (100 μ g) were separated on 10% SDS-polyacrylamide gels and then transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore Corp.). The membranes were incubated with anti-CXCR4, anti-Bcl-2, and anti- β -actin antibodies. The membranes were subsequently incubated with horseradish-peroxidase-conjugated secondary antibody and visualized by CL (GE Healthcare). The density of the blots was quantified using image-analysis software after scanning the image (Photoshop, version 7.0; Adobe Systems). The optical densities of each band were calculated and standardized based on the density of the β -actin band.

SH-SY5Y cell culture and oxidative insult in vitro

SH-SY5Y cells were obtained from the Bioresource Collection and Research and used for *in vitro* studies. SH-SY5Y cells were incubated in DMEM supplemented with 10% fetal bovine serum. All cells were maintained at 37°C in a humidified 5% CO₂ and 95% air incubator. For the oxidative insult, PQ was diluted in culture medium to the final concentration of 100, 500, or 1,000 μ M immediately before use. Cells were seeded on coverslips for 24 h and treated with various concentrations of PQ for another 24 h. The protocol utilized for TUNEL staining was as described previously. Cells were counterstained with DAPI. The TUNEL assay results were examined by immunofluorescence microscopy.

Effects of SDF-1 α on cell apoptosis in PQ-induced SH-SY5Y cells

Human recombinant SDF-1 α (Peprotech) was dissolved in PBS. SH-SY5Y cells were pretreated with each concentration of SDF-1 α (0, 0.1, 1, 10, or 50 ng/mL) for 30 min.

After SDF-1 α treatment, cells were exposed to 1,000 μ M PQ for 24 h at 37°C and then fixed with 4% paraformaldehyde for TUNEL analysis.

SH-SY5Y cells were pretreated with 10 ng/mL SDF-1 α for 30 min and then exposed to 1,000 μ M PQ for 24 h. Western blot analysis was used to determine the expression of the antiapoptotic protein Bcl-2, as described previously.

Assessment of tumor formation after subretinal transplantation of non-c-Myc iPSCs

To assess whether iPSCs lacking exogenous *c-Myc* reduced the incidence of tumorigenesis, either 3 genes (Oct4/Sox2/Klf4) or 4 genes (Oct4/Sox2/Klf4/*c-Myc*) were delivered using a lentiviral vector. The tumorigenic ability of the different iPSC grafts was compared after PQ injection and intravitreal cell transplantation for 6 months after treatment. Retinal sections with H&E staining were performed to evaluate the incidence of teratoma formation.

Statistical analysis

All data are shown as means \pm standard errors of means. Statistical analysis was performed using 1-way analysis of variance (ANOVA) followed by Tukey's *post hoc* analysis for multiple comparisons with the computer software Statistical Package for Social Science (SPSS 15.0; SPSS, Inc.). Differences in the means between 2 groups were assessed with an unpaired, 2-tailed *t*-test. A value of $P < 0.05$ was considered significant.

Results

Effects of non-c-Myc iPSC transplantation on ERGs

Three days after injection of 0.5 mM PQ, the b-wave ratio on the ERG was reduced in each treatment group. There was no significant difference in b-wave ratio between the non-*c-Myc* iPSC-treated, PQ-injected, and MEF-treated groups 3 days after transplantation ($P > 0.05$, 1-way ANOVA). However, the non-*c-Myc* iPSC-treated group enhanced the recovery of the b-wave ratio at 7, 14, 21, and 28 days after transplantation. The b-wave ratio reached statistical significance in the iPSC-treated group after 7, 14, 21, and 28 days compared with the PQ-injected and MEF-treated groups ($P < 0.05$, 1-way ANOVA; $N = 10$ in each group) (Fig. 1A, B).

Effects of non-c-Myc iPSC transplantation on retinal morphology and outer nuclear cell number

There was thinning of the INL and ONL, and folds in the ONL at day 14 in the retinas of PQ-injected and MEF-treated groups. Non-*c-Myc* iPSC-treated retinas showed better preservation of the INL and ONL, with less prominent folds in the ONL (Fig. 1C). Significantly more total number of cells in the GCL, INL, and ONL was counted in non-*c-Myc* iPSC-treated retinas than in MEF-treated or PQ-injected retinas ($P < 0.05$, 1-way ANOVA; $N = 3$ in each group) (Fig. 1D).

Effects of non-c-Myc iPSC transplantation on retinal apoptosis

More TUNEL-positive cells in the GCL, INL, and ONL were noted in MEF-treated and PQ-injected retinas, while

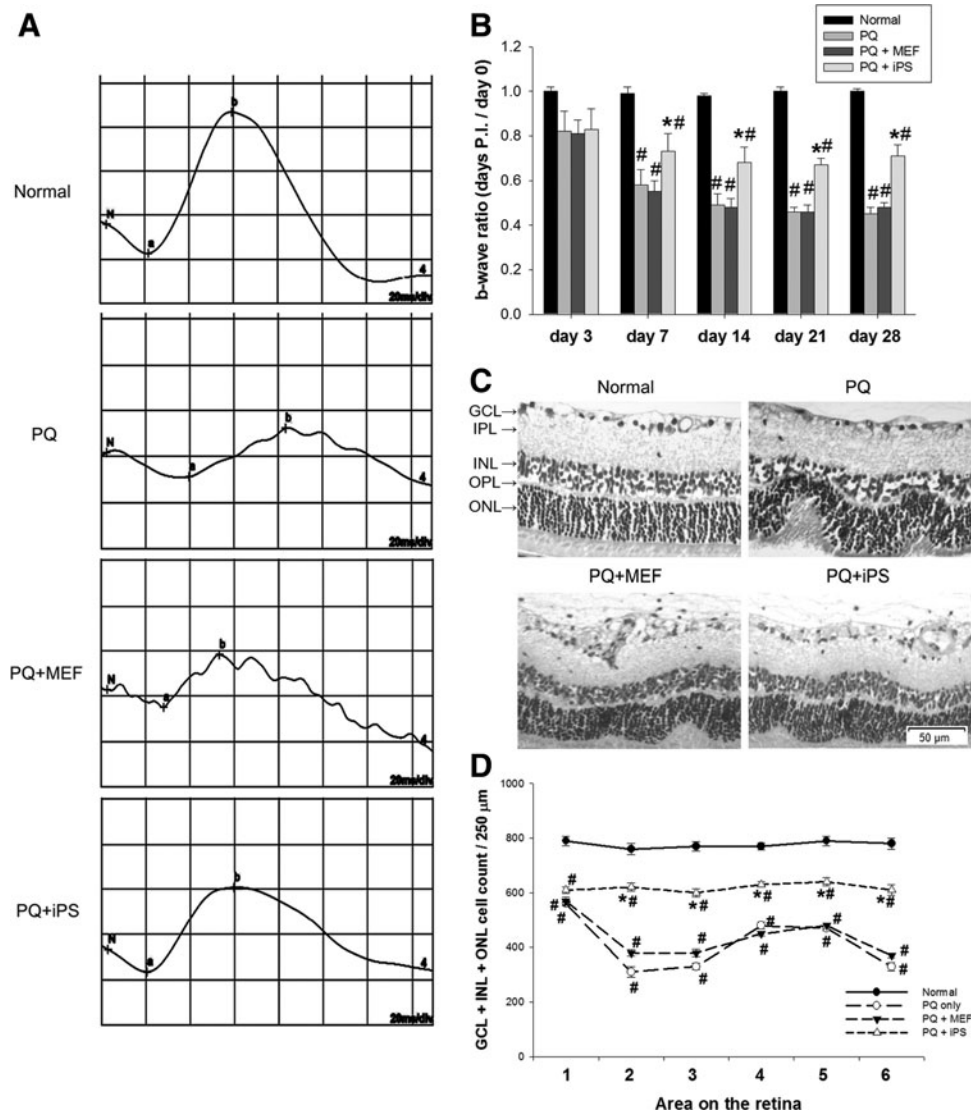


FIG. 1. Effects of subretinal transplantation of non-*c-Myc* induced pluripotent stem cells (iPSCs) on electroretinogram (ERG) b-wave amplitudes, retinal morphology, and the number of outer nuclear layer (ONL) cells. **(A)** Typical individual ERG recordings from the 4 groups at day 14 after treatment. **(B)** Mean ERG b-wave amplitude was measured at days 3, 7, 14, 21, and 28 after treatment. The b-wave ratio was defined as the b-wave amplitude of the right eye divided by the b-wave amplitude of the left eye at the specific time point. The fold of the b-wave ratio represents the b-wave ratio at days 3, 7, 14, 21, or 28 divided by the b-wave ratio at day 0 ($N=10$ in each group). **(C)** Retinal sections were stained with hematoxylin and eosin (H&E) at day 14 after treatment. **(D)** The total number of cells in the ganglion cell layer (GCL), inner nuclear layer (INL), and ONL at day 14 was counted along ventral retinal sections from one margin to the other, and labeled as areas 1 to 6, each 250 μm in length along the section. The result is expressed as the cell number per 250 μm of retina ($N=3$ in each group). Bars show the mean \pm SEM. The scale bar represents 50 μm . The GCL, inner plexiform layer (IPL), INL, outer plexiform layer (OPL), and ONL are labeled in the micrograph. * $P < 0.05$ compared with the “PQ” and “PQ+MEF” groups; # $P < 0.05$ compared with the “normal” group; by 1-way ANOVA. ANOVA, analysis of variance; MEF, murine embryonic fibroblast; PQ, paraquat; SEM, standard error of mean.

only sparse TUNEL-positive cells were noted in non-*c-Myc* iPSC-treated retinas at day 7. After counting the number of apoptotic cells, there were statistically fewer apoptotic cells in non-*c-Myc* iPSC-treated retinas than in MEF-treated and PQ-injected retinas ($P < 0.05$, 1-way ANOVA; $N=3$ in each group) (Fig. 2A).

Similarly, non-*c-Myc* iPSC-treated retinas had significantly fewer caspase 3-positive cells in the retinas compared with MEF-treated and PQ-injected retinas at day 7 ($P < 0.05$, 1-way ANOVA; $N=3$ in each group) (Fig. 2B).

Effects of non-*c-Myc* iPSC transplantation on retinal oxidative damage

We examined the retinal oxidative damage to proteins, lipids, and DNA by staining retinal sections with nitrotyrosine, acrolein, and 8-OHdG at day 14 after treatment. PQ injection resulted in an increase in immunohistochemical staining for nitrotyrosine, acrolein, and 8-OHdG in the retinas. Non-*c-Myc* iPSC-treated retinas showed decreased staining for nitrotyrosine, acrolein, and 8-OHdG, especially

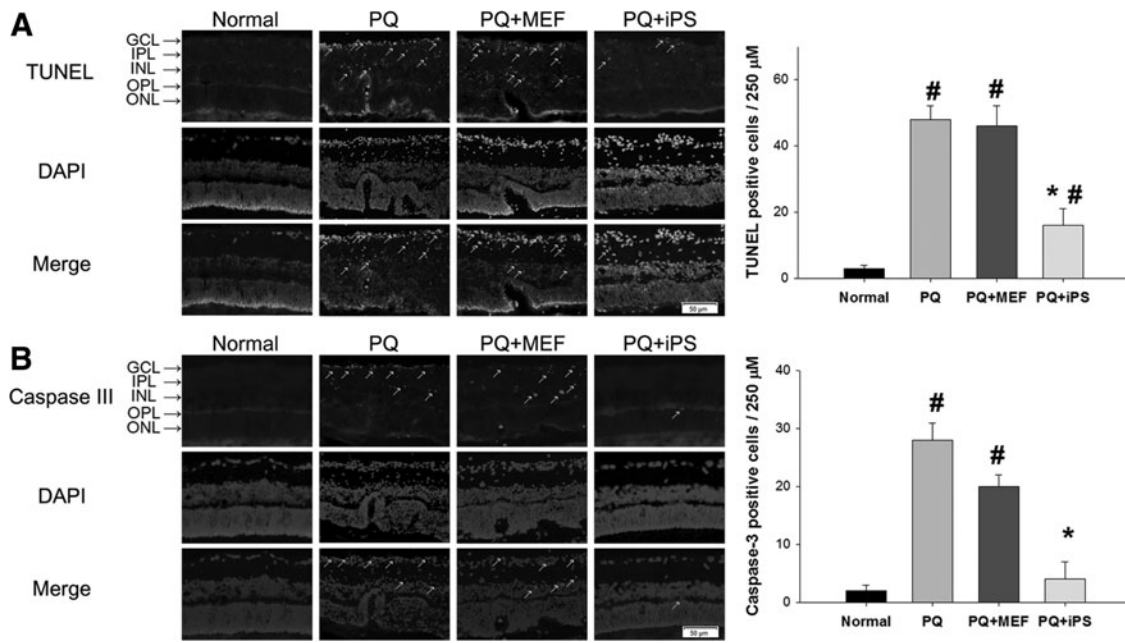


FIG. 2. Effects of non-*c-Myc* iPSC transplantation on retinal apoptosis. Retinal sections were stained with TUNEL and caspase 3, and the mean number of TUNEL- or caspase 3-positive retinal cells per 250 μ m section of retina was counted at day 7. **(A)** More TUNEL-positive cells in GCL, INL, and ONL were observed in MEF-treated and PQ-injected retinas (arrows). There were statistically fewer TUNEL-positive, counted cells in non-*c-Myc* iPSC-treated retinas than in MEF-treated and PQ-injected retinas. **(B)** Few caspase 3-positive cells in the INL of the retina were noted in iPSC-treated retinas. There were also statistically significantly less caspase 3-positive cells in iPSC-treated retinas than in MEF-treated or PQ-injected retinas (arrows) ($N=3$ in each group). Bars show the mean \pm SEM. The scale bar represents 50 μ m. $*P<0.05$ compared with the “PQ” and “PQ+MEF” groups; $^{\#}P<0.05$ compared with the “normal” group; by 1-way ANOVA. TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotinide end labeling.

in the GCL and ONL, compared with PQ-injected retinas and MEF-treated retinas (Fig. 3A–C).

ELISA analysis showed that the levels of MDA and 8-OHdG, which represent oxidative damage to lipids and DNA, were significantly reduced in the iPSC-treated retinas at day 14 compared with the PQ-injected or MEF-treated groups ($P<0.05$ and $P<0.05$, respectively, 1-way ANOVA; $N=3$ in each group) (Fig. 3D, E).

Effects of non-*c-Myc* iPSC transplantation on retinal free-radical production

The generation of ROS in the retinas was examined using lucigenin- and luminol-enhanced CL. Non-*c-Myc* iPSC-treated retinas showed statistically significant decreases in mean luminol- and lucigenin-enhanced CL at days 7, 14, and 21 compared with MEF-treated or PQ-injected retinas ($P<0.05$, 1-way ANOVA; $N=3$ in each group) (Fig. 4A, B).

Effects of non-*c-Myc* iPSC transplantation on the activity of antioxidant enzymes

SOD and catalase (CAT) are the most important enzymes of the antioxidant defense system. As shown in Fig. 4C and D, intravitreal injection of PQ resulted in a significant decrease in the activity of SOD and CAT enzymes compared with the normal control ($P<0.05$, 2-tailed Student's *t*-test; $N=3$ in each group). However, subretinal transplantation of iPSCs significantly attenuated the decrease of SOD and CAT activity at days 7, 14, and 21 compared with MEF-treated or PQ-only groups ($P<0.05$, 2-way ANOVA; $N=3$ in each group).

Non-*c-Myc* iPSC transplantation increased SDF-1 α expression in oxidative-damaged retinas

In ELISA-based quantitative measurements, the levels of SDF-1 α started to increase in the non-*c-Myc* iPSC-treated retinas at day 3. The levels reached a maximum at day 7 and remained elevated on day 21. There was a statistically significant difference in the amount of SDF-1 α in non-*c-Myc* iPSC-treated retinas compared with MEF-treated and PQ-only retinas at days 3, 7, 14, and 21 ($P<0.05$, 2-way ANOVA; $N=3$ in each group) (Fig. 5A). Western blot analysis demonstrated a significant increase in the expression of CXCR4 in the non-*c-Myc* iPSC-treated retinas compared with the normal or MEF-treated or PQ-injected retinas at day 7 ($P<0.05$, 1-way ANOVA; $N=3$ in each group) (Fig. 5B).

In the iPSC-treated retinas, the intensity of SDF-1 α immunoreactivity was increased throughout the retinas, especially in the GCL at day 3 (Fig. 5C). Corresponding to the upregulation of SDF-1 α immunoreactivity, CXCR4 immunoreactivity was elevated in iPSC-treated retinas, with abundant fluorescence staining in the GCL (Fig. 5D). Retinal sections from the normal group showed only background fluorescence staining for CXCR4.

SDF-1 α protects SH-SY5Y cells from oxidative stress in vitro

In the previous results, we have shown that SDF-1 α could be an important mediator to modulate the beneficial effects of iPSCs. We then investigated whether SDF-1 α

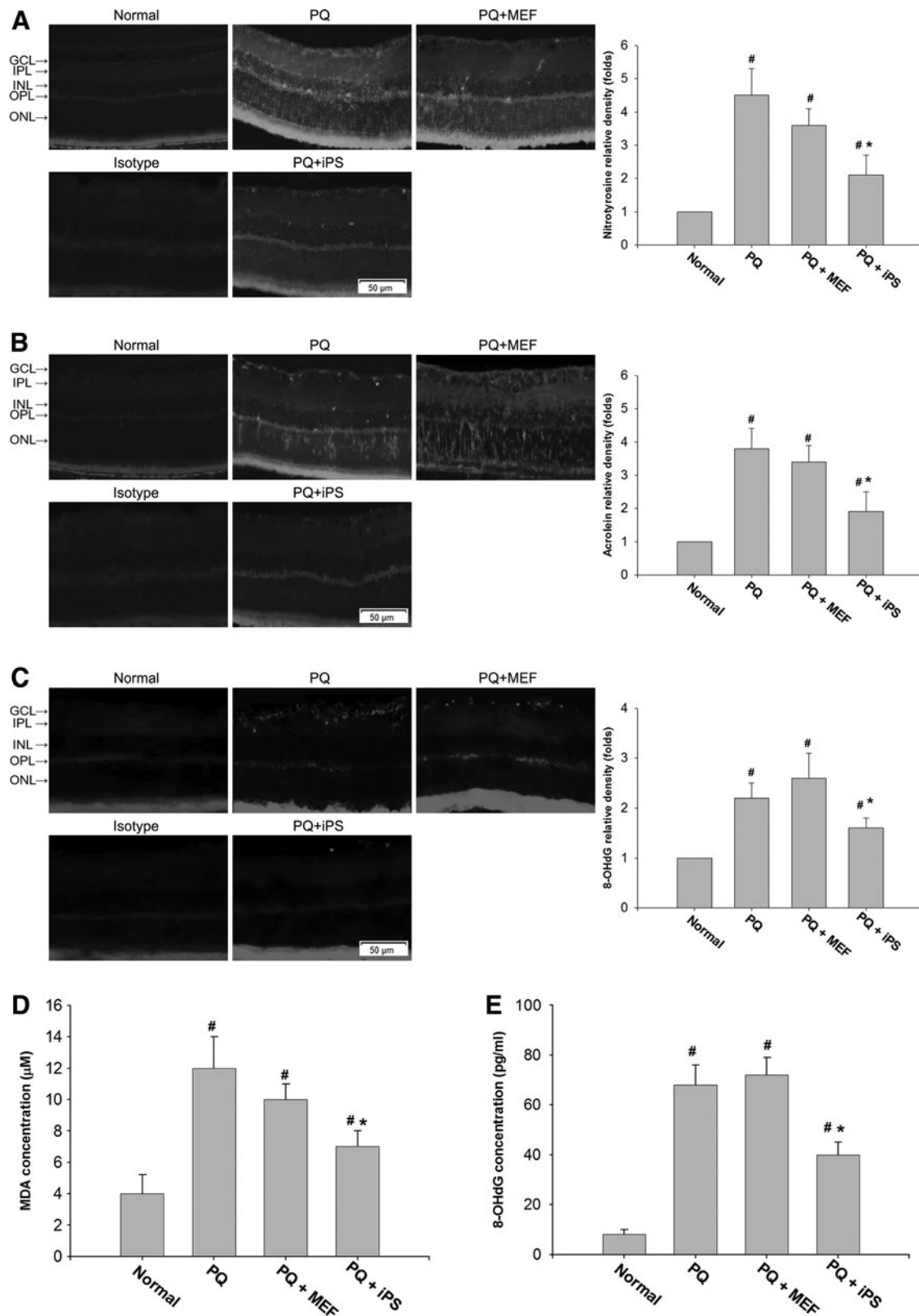


FIG. 3. The effects of non-*c-Myc* iPSC transplantation on oxidative damages to the retina. Retinal oxidative stress in each group was detected by immunohistochemical staining of (A) nitrotyrosine, (B) acrolein, and (C) 8-OHdG at day 14 after transplantation. PQ injection resulted in an increase in immunohistochemical staining for nitrotyrosine, acrolein, and 8-OHdG in the retinas. Non-*c-Myc* iPSC-treated retinas showed decreased staining for nitrotyrosine, acrolein, and 8-OHdG compared with PQ-injected or MEF-treated retinas. Specimens stained with IgG isotype antibody as the primary antibody were used as negative controls. The levels of (D) MDA and (E) 8-OHdG in the retinas, which represent oxidative damage to lipids and DNA, were examined at day 14 by ELISA. iPSC-treated retinas showed significantly reduced levels of MDA and 8-OHdG at day 14 compared with the PQ-injected or MEF-treated groups ($N=3$ in each group). Bars show the mean \pm SEM. The scale bar represents 50 μm . [#] $P < 0.05$ compared with the PQ- or MEF-treated groups; ^{**} $P < 0.05$ compared with the normal group; by 1-way ANOVA.

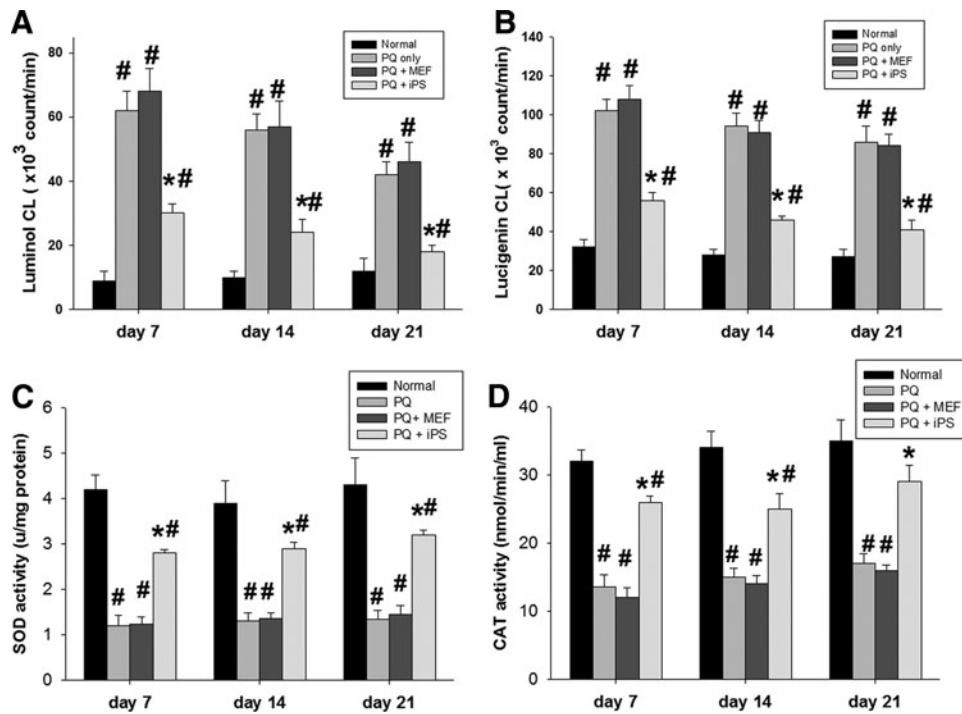


FIG. 4. Effects of non-*c-Myc* iPSC transplantation on retinal reactive oxygen species and antioxidant enzyme activity. (A) iPSC-treated retinas showed a statistically significant reduction of luminol- and (B) lucigenin-enhanced chemiluminescence compared with PQ-injected retinas and MEF-treated retinas at days 7, 14, and 21. (C) Untreated retinas and MEF-treated retinas showed significantly decreased activity of SOD and (D) CAT enzymes 7 days after injury compared with iPSC-treated retinas. iPSC treatment significantly attenuated the decrease of SOD and CAT activity at days 7, 14, and 21 after transplantation ($N=3$ in each group). Bars show the mean \pm SEM. The scale bar represents 50 μm . * $P < 0.05$ compared with the “PQ” and “PQ + MEF” groups; # $P < 0.05$ compared with the “normal” group; by 2-way ANOVA. CAT, catalase; SOD, superoxide dismutase.

can protect neuronal cells from oxidative damage *in vitro*. Treatment with 1, 10, and 50 ng/mL of SDF-1 α significantly reduced PQ-induced apoptotic cell death on SH-SY5Y cells in a dose-dependent manner compared with treatment with PBS ($P < 0.05$, 2-way ANOVA; $N=3$ in each group) (Fig. 6A).

Expression of the antiapoptotic protein Bcl-2 was significantly upregulated when cells were exposed to PQ and SDF-1 α pretreatment ($P < 0.05$, 1-way ANOVA; $N=3$ in each group) (Fig. 6B).

Effects of non-*c-Myc* iPSC transplantation on the expression of bFGF, CNTF, and BDNF in the retina

Immunohistochemistry showed that bFGF immunoreactivity was significantly elevated in the GCL and INL of non-*c-Myc* iPSC-treated retinas compared with normal, PQ-injected, and MEF-treated retinas at day 14 after transplantation (Fig. 7A). Intravitreal PQ injection increased CNTF and BDNF immunoreactivity because more CNTF and BDNF immunoreactivity was found in PQ-only, MEF-treated, and non-*c-Myc* iPSC-treated retinas compared with normal retinas. However, the non-*c-Myc* iPSC-treated retinas did not statistically significantly increase CNTF and BDNF immunoreactivity compared with PQ-injected and MEF-treated retinas (Fig. 7B, C).

In ELISA-based quantitative measurements, the amount of bFGF started to increase in the non-*c-Myc* iPSC-treated retinas at day 3 but did not reach statistical significance com-

pared with the MEF-treated or PQ-only retinas ($P=0.09$, 2-way ANOVA; $N=5$ in each group). However, there was significantly higher bFGF in the non-*c-Myc* iPSC-treated retinas than in the normal, MEF-treated, and PQ-injected retinas at 7, 14, and 21 days after transplantation ($P < 0.05$, 2-way ANOVA; $N=5$ in each group) (Fig. 7D). The amount of CNTF expression was increased in non-*c-Myc* iPSC-treated, MEF-treated, and PQ-only retinas 14 days after transplantation. However, there were no significant differences between these groups ($P > 0.05$, 1-way ANOVA; $N=5$ in each group) (Fig. 7E). Similarly, no significant difference in BDNF expression was found among normal, non-*c-Myc* iPSC-treated, MEF-treated, and PQ-injected retinas at 3, 7, 14, and 21 days after transplantation ($P > 0.05$, 2-way ANOVA; $N=5$ in each group) (Fig. 7F).

Localization of transplanted non-*c-Myc* iPSCs

In a separate study, transplanted non-*c-Myc* iPSCs were prelabeled with CM-DiI to track the transplanted cells. Additionally, immunohistochemistry against neurofilament-M was performed to study whether transplanted non-*c-Myc* iPSCs could differentiate into neuronal cells. We found that many transplanted cells survived and mainly remained in the subretinal space at 14 days after transplantation. Some transplanted cells expressed neurofilament-M, indicating that these cells could differentiate into neuronal cells in the subretinal space (Fig. 8A). By 28 days after transplantation, the transplanted non-*c-Myc* iPSCs still remained in the subretinal space. These cells

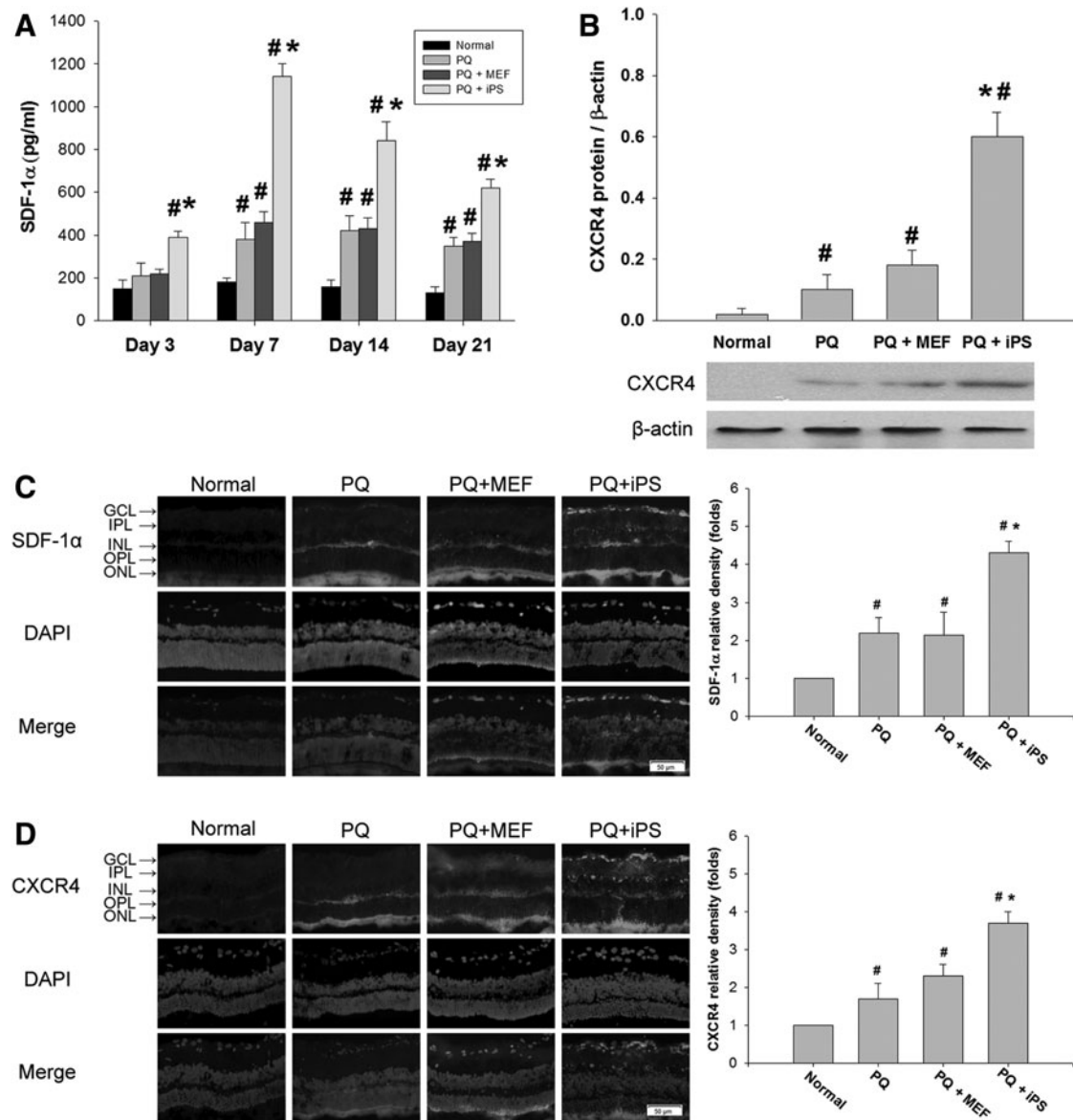


FIG. 5. Effects of non-*c-Myc* iPSC transplantation on the expression of SDF-1 α and CXCR4 in retinas. (A) The concentration of SDF-1 α in each treatment group was quantified by ELISA at days 3, 7, 14, and 21 ($N=3$ in each group). (B) The expression of CXCR4 protein in retinas at day 7 was measured by western blot. Immunohistochemical staining against (C) SDF-1 α and (D) CXCR4 in retinas was performed 14 days after transplantation. SDF-1 α and CXCR4 immunoreactivity was significantly increased in the GCL of iPSC-treated retinas. Bars show the mean \pm SEM. The scale bar represents 50 μ m. * $P < 0.05$ compared with the “PQ” and “PQ+MEF” groups; # $P < 0.05$ compared with the “normal” group; by 2-way ANOVA. SDF, stromal cell-derived factor.

could also differentiate and expressed neurofilament-M (Fig. 8B).

Assessment of tumor formation after subretinal transplantation of non-*c-Myc* iPSCs

No tumor formation was observed in 10 rats receiving the 3-gene iPSC transplantation by 6 months after treatment. Two rats that received the 4-gene iPSC developed tumor loci (Table 2).

Discussion

In this study, we demonstrated that subretinal transplantation of non-*c-Myc* iPSCs enhanced the recovery of retinal

function and improved histopathologic changes that were caused by intravitreal PQ injection. Further, non-*c-Myc* iPSCs increased the activities of the antioxidant enzymes SOD and catalase and decreased ROS levels in the retinas, which may be a mechanism for protecting the retinas against oxidative damage and apoptotic cell death. Remarkably, non-*c-Myc* iPSC transplantation increased retinal SDF-1 α levels at time points preceding functional improvement, followed by enhanced bFGF expression in later periods. *In vitro* studies confirmed that SDF-1 α contributed to the protection of retinal cells from oxidative damage. These findings indicated that the therapeutic mechanisms of non-*c-Myc* iPSCs were partially due to reduced retinal oxidative stress and the paracrine secretion of trophic factors SDF-1 α

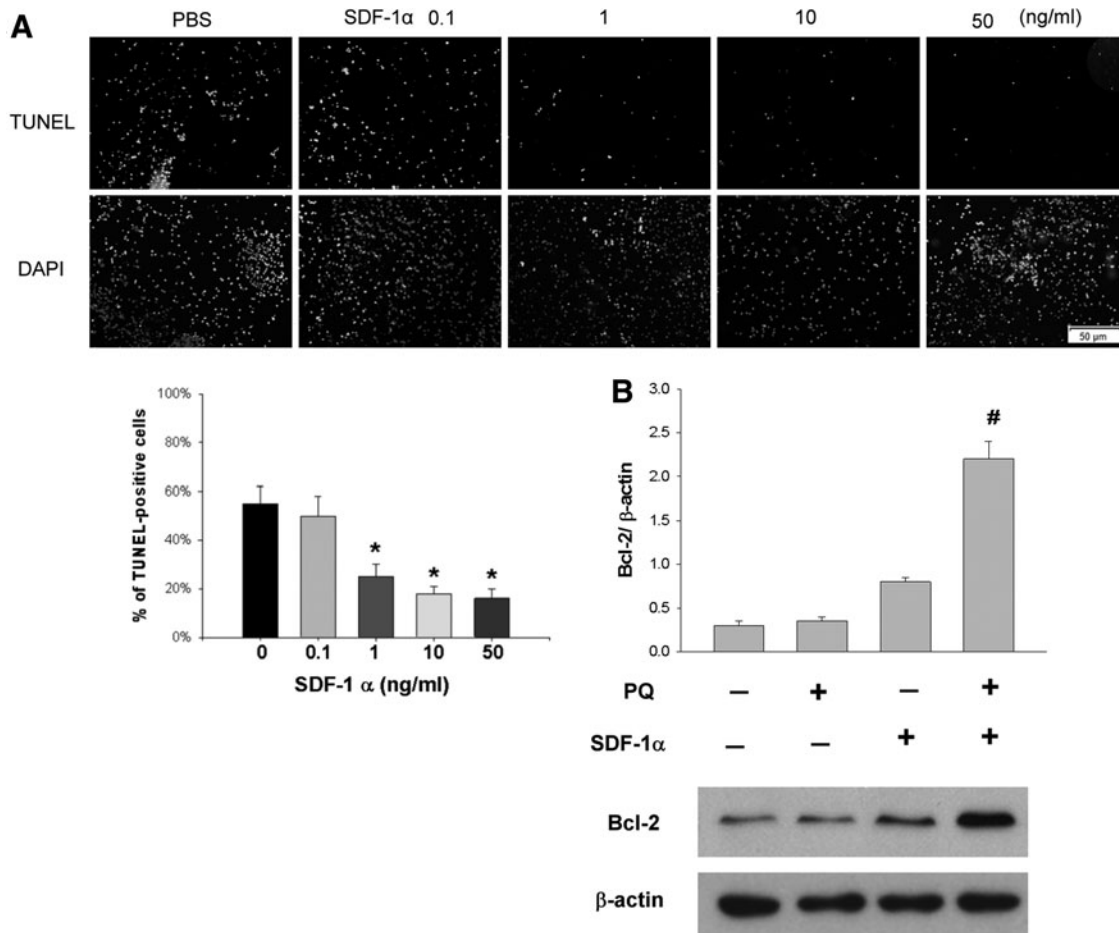


FIG. 6. Antiapoptotic effects of SDF-1 α on SH-SY5Y cells *in vitro*. **(A)** SH-SY5Y cells were pretreated with 1, 10, or 50 ng/mL SDF-1 α for 30 min and then exposed to 1,000 μ M PQ for 24 h. Treatment with 1, 10, and 50 ng/mL of SDF-1 α significantly reduced apoptotic cell death compared with treatment with PBS ($N=3$ in each group). * $P < 0.05$ compared with treatment with 0 ng/mL; by 2-way ANOVA. **(B)** Expression of the antiapoptotic protein Bcl-2 was significantly upregulated when cells were exposed to PQ and SDF-1 α pretreatment. Bars show the mean \pm SEM. The scale bar represents 50 μ m. [#] $P < 0.05$ compared with normal control; by 1-way ANOVA. PBS, phosphate-buffered saline.

and bFGF. Our results demonstrated the therapeutic potential of non-*c-Myc* iPSC transplantation in treating oxidative-damage-induced retinal diseases.

It has been demonstrated that SDF-1/CXCR4 recruits bone-marrow-derived cells to neovascularization and regeneration sites in the heart, liver, and eye.^{22,23} However, apart from stem cell mobilization, increasing evidence has revealed that SDF-1 α activates cell survival signaling and exerts antiapoptotic and neuroprotective effects.²⁴ Wang et al. showed that intravenous administration of mesenchymal stem cells provides neuroprotection through the antiapoptotic effects of SDF-1 α on a parkinsonian model of rats.²⁵ Otsuka et al. showed that SDF-1 protected photoreceptor cells in a rat model of retinal detachment.²⁶ In this study, we demonstrated that non-*c-Myc* iPSCs increased the production of SDF-1 α in retinas early, at day 3 after transplantation. The significant increases in SDF-1 α detected at an early stage after transplantation revealed the important roles of SDF-1 α in angiogenesis, antiapoptosis, and the sparing of damaged retinal cells in PQ-induced retinal damage. The *in vitro* study confirmed that treatment with SDF-1 α effectively prevented PQ-treated SH-SY5Y cells from apoptosis by upregulating the antiapoptotic protein

Bcl-2. Taken together, our data provide evidence that SDF-1 α plays an important regulatory role in facilitating the repair of retinal oxidative damage after iPSC transplantation.

Our results showed that transplanted non-*c-Myc* iPSCs survived mainly in the subretinal space and expressed a neuronal marker at day 14 after transplantation. However, the rescue of retinal function with non-*c-Myc* iPSC treatment was detected on day 7. These results suggested that the therapeutic benefit of non-*c-Myc* iPSC transplantation was not due to the differentiation of non-*c-Myc* iPSCs into neuronal cells. Alternatively, we found that non-*c-Myc* iPSC treatment significantly increased the expression of bFGF in retinas from days 7 to 21, concurrent with periods of functional improvement. These results implied that bFGF may also contribute to the functional improvements observed after iPSC transplantation. bFGF is a multifunctional protein that exerts neuroprotective and beneficial effects on functional outcomes in animal studies of oxidative damage.^{27,28} O'Driscoll et al. found that intravitreal bFGF injection into mice prior to light damage almost completely protected photoreceptors from light-induced degeneration.²⁹ Xiao et al. demonstrated that bFGF protects the heart against ischemia/reperfusion-induced oxidative damage under diabetic

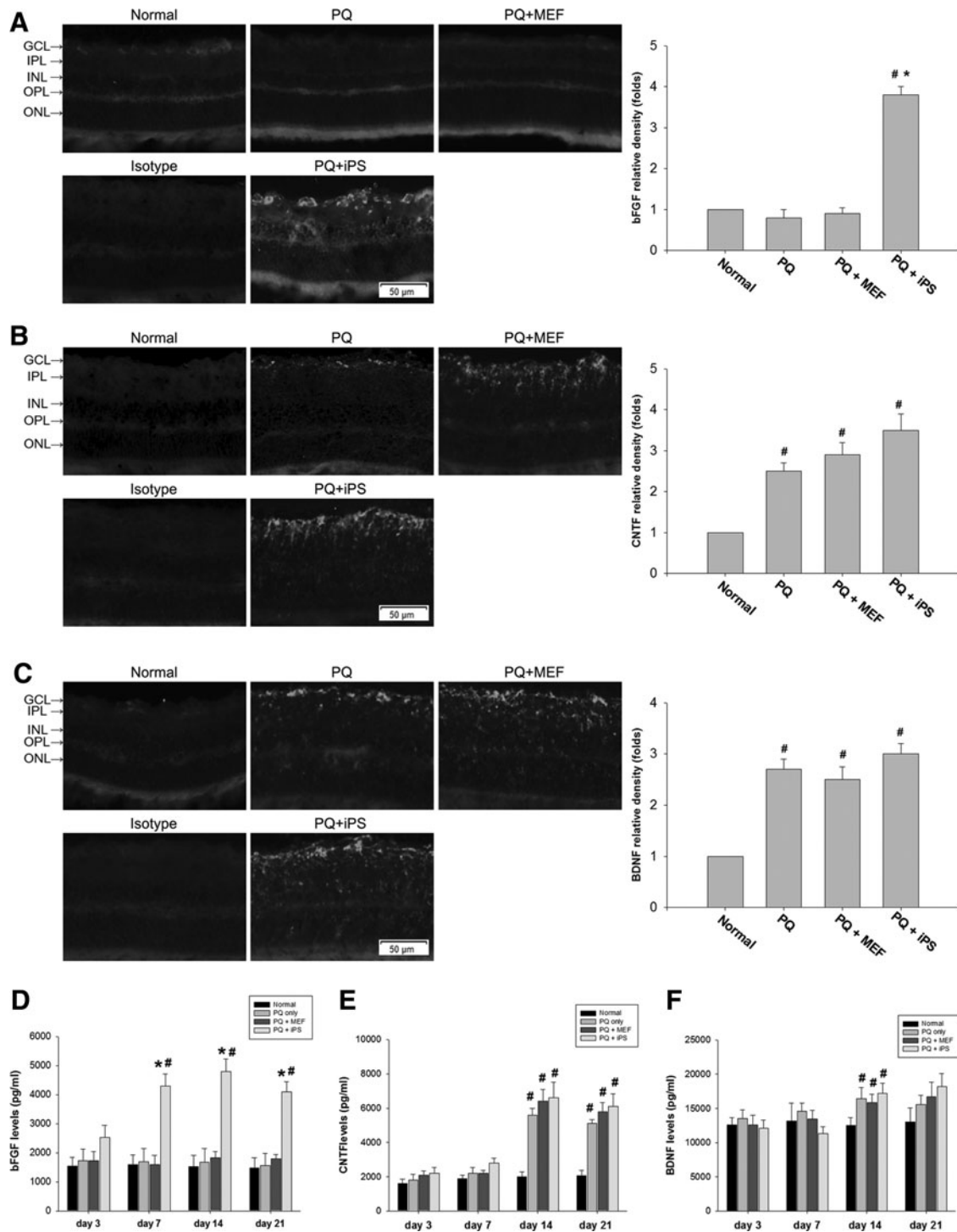


FIG. 7. Immunohistochemical and ELISA analyses of the neurotrophic factors BDNF, CNTF, and bFGF in the retinas of non-*c-Myc* iPSC-transplanted and control rats. Immunohistochemical staining against (A) bFGF, (B) CNTF, and (C) BDNF was performed 14 days after transplantation. Specimens stained with IgG isotype antibody as the primary antibody were used as negative controls. bFGF immunoreactivity was significantly elevated in the INL and GCL of non-*c-Myc* iPSC-treated retinas ($N=3$ in each group). The expression of (D) bFGF, (E) CNTF, and (F) BDNF in each treatment group was quantified by ELISA analysis at days 3, 7, 14, and 21. The amount of bFGF expression was significantly higher in non-*c-Myc* iPSC-treated retinas than in the normal, MEF-treated, and PQ-injected retinas at 7, 14, and 21 days after transplantation ($N=5$ in each group). Bars show the mean \pm SEM. The scale bar represents 50 μ m. * $P < 0.05$ compared with the “PQ” and “PQ+MEF” groups; # $P < 0.05$ compared with the “normal” group; by 2-way ANOVA. BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; CNTF, ciliary neurotrophic factor.

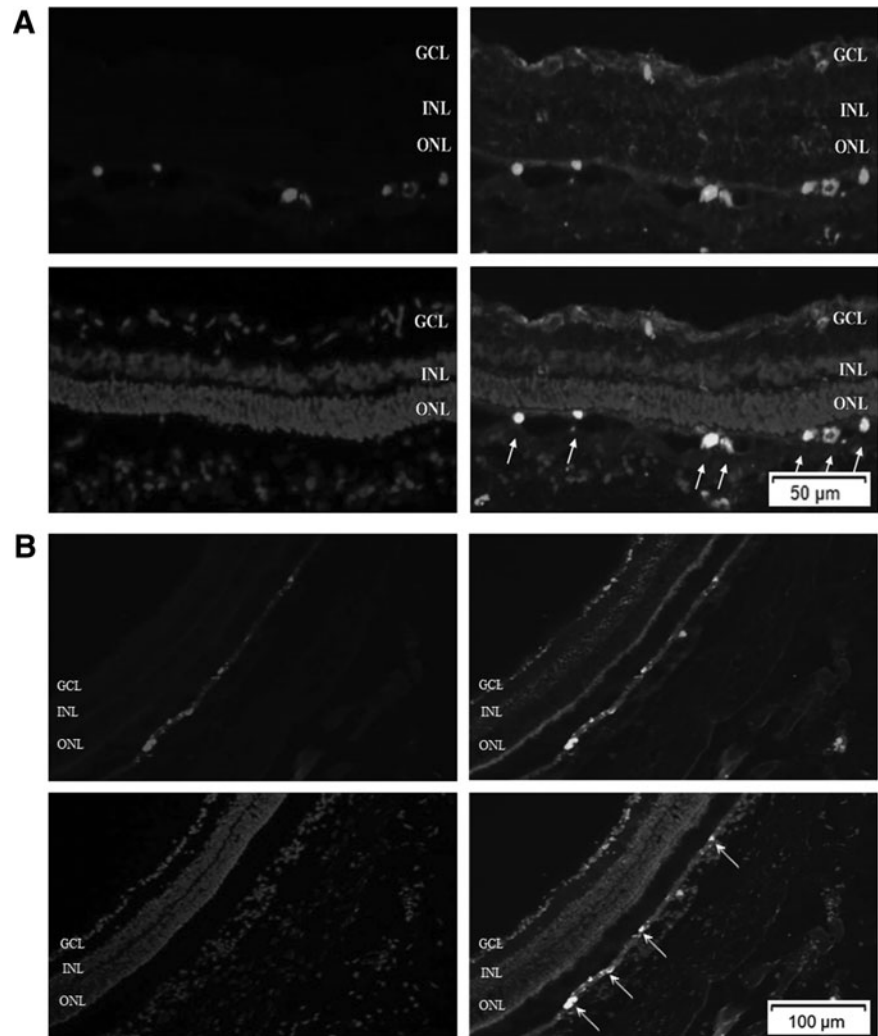


FIG. 8. Localization of transplanted non-*c-Myc* iPSCs 14 and 28 days after transplantation. The iPSCs were pre-labeled with the red fluorescent dye CM-DiI. The retinal sections were stained with rabbit polyclonal anti-neurofilament M antibody and then GFP-conjugated anti-rabbit secondary antibody. DAPI was used to label the nucleus. **(A)** At day 14 after transplantation, many transplanted cells survived and remained in the sub-retinal space. Some transplanted cells showed positive staining for neurofilament-M. **(B)** At day 28 after transplantation, transplanted non-*c-Myc* iPSCs still remained in the subretinal space and showed positive staining for neurofilament-M ($N=3$ in each group).

conditions.³⁰ In a previous study, we have shown that cultured iPSCs can secrete bFGF *in vitro*.³¹ In this study, we demonstrated that the levels of bFGF did not significantly increase at day 3 but did reach significance later, at days 7, 14, and 21, while SDF-1 α was upregulated early, at day 3 after iPSC transplantation. Shyu et al. demonstrated that SDF-1 α exerted its neuroprotective effects by increasing the synthesis or release of neurotrophic factors in a rat model of stroke.³² Further investigation is warranted to clarify the precise mechanisms between SDF-1 α and the neurotrophic factor bFGF.

ROS are known to be generated during PQ-induced retinal damage and may be pivotal mediators of the ensuing pathological complications. ROS produced in the body are

scavenged by antioxidant enzymes, such as SOD and CAT. A recent study has shown that iPSCs have defense mechanisms against ROS, similar to the elevated antioxidant enzyme expression and increased strand break repair capacity of embryonic stem cells.³³ In this study, we evaluated the generation of ROS in the retina by using luminol and lucigenin CL assays. Luminol detects H₂O₂, OH⁻, hypochlorite, peroxynitrite, and lipid peroxy radicals, whereas lucigenin is particularly sensitive to superoxide radicals.³⁴ We found that retinal-injected PQ significantly increased luminol and lucigenin CL levels and decreased the activities of SOD and CAT in the retina, whereas non-*c-Myc* iPSC treatment effectively decreased both the luminol and lucigenin CL levels and enhanced antioxidant enzyme activity.

TABLE 2. INCIDENCE OF TUMOR FORMATION IN RECIPIENTS OF 4-GENE AND 3-GENE INDUCED PLURIPOTENT STEM CELLS AND MURINE EMBRYONIC FIBROBLASTS 6 MONTHS AFTER TRANSPLANTATION

Group tumor formation	1 month	2 month	3 month	4 month	5 month	6 month
iPSC (4-genes)	0/10	0/10	2/10	2/10	2/10	2/10
Non- <i>c-Myc</i> iPSCs (3-genes)	0/10	0/10	0/10	0/10	0/10	0/10
MEFs	0/10	0/10	0/10	0/10	0/10	0/10

iPSC, induced pluripotent stem cell; MEF, murine embryonic fibroblast.

Moreover, we examined the oxidative damage to lipids, proteins, and DNA in the retina by staining for acrolein, nitrotyrosine, and 8-OHdG. In accordance with the decrease in ROS production, iPSC treatment also significantly decreased oxidative damage to these macromolecules and thus reduced the number of apoptotic cells in the retina. Taken together, our data indicated that non-*c-Myc* iPSCs are also endowed with antioxidative abilities, which contribute to the neuroprotection of retinal cells from PQ injury.

In this study, we have demonstrated that transplantation of about 100 non-*c-myc* iPSCs can protect the retina from oxidative damage in an animal model. Carr et al. demonstrated that transplantation of about 100 iPSC-derived RPE into the subretinal space of RCS rats is sufficient to phagocytose photoreceptor outer segments and facilitate the short-term maintenance of photoreceptors.³⁵ However, we only followed the efficacy of non-*c-myc* iPSC treatment for 28 days. Further investigation is needed to determine the long-term efficacy of non-*c-myc* iPSCs, including the rescue of retinal function, antioxidative effects, and sustained secretion of neuroprotective factors by the engrafted cells. In addition, the formation of teratomas has been a major problem for iPSC transplantation. Despite this concern, in the present study, we did not implant iPSCs after differentiation into other cell types. This is because PQ-induced retinal injury causes widespread damage to several types of retinal cells, including retinal ganglion cells, amacrine cells, and bipolar cells.¹⁸ Therefore, transplantation of a single type of iPSC-derived retinal cell seemed insufficient to rescue all the cell types injured in this disease. Further, other evidence has indicated that the therapeutic effects of stem cell transplantation may be attributed to paracrine effects, but not to tissue regeneration through stem cell differentiation.^{36,37}

The oncogene *c-Myc* has been shown to substantially contribute to tumor formation.^{38,39} Dysregulated expression of *c-Myc* occurs in a wide range of human cancers and is often associated with poor prognosis, indicating a key role for this oncogene in tumor progression.⁴⁰ Our previous study demonstrated that suppression of *c-Myc* expression effectively blocked teratoma formation in rats transplanted with docosahexaenoic-acid-treated iPSCs 4 months after transplantation.⁴¹ Therefore, exclusion of *c-Myc* would be a feasible way to reduce the incidence of tumor formation. In the present study, no tumor formation was observed in the recipients of non-*c-myc* iPSCs over 6 months. However, due to the safety concerns in clinical applications, nonviral vector delivery and reprogramming without pro-oncogenes would be a good strategy for future studies.

Conclusions

In this study, we demonstrated that non-*c-myc* iPSC transplantation attenuated the severity of the physiologic and histopathologic impairment caused by PQ-induced retinal damage. These protective effects were mediated by increased production of SDF-1 α and bFGF and regulation of oxidative stress responses. Thus, non-*c-Myc* iPSC transplantation may be a potential resource for stem-cell-based therapy in oxidative retinal diseases.

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Author Disclosure Statement

All authors have no conflicts of interest in this work.

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