Neuroprotective Effects of Intravitreal Mesenchymal Stem Cell Transplantation in Experimental Glaucoma

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Purpose. Retrograde neurotrophic factor transport blockade has been implicated in the pathophysiology of glaucoma. Stem cell transplantation appears to ameliorate some neurodegenerative conditions in the brain and spinal cord, in part by neurotrophic factor secretion. The present study was conducted to determine whether local or systemic bone marrow-derived mesenchymal stem cell (MSC) transplantation can confer neuroprotection in a rat model of laser-induced ocular hypertensive glaucoma.

Methods. MSCs were isolated from the bone marrow of adult wild-type and transgenic rats that ubiquitously express green fluorescent protein. MSCs were transplanted intravitreally 1 week before, or intravenously on the day of, ocular hypertension induction by laser photocoagulation of the trabecular meshwork. Ocular MSC localization and integration were determined by immunohistochemistry. Optic nerve damage was quantified by counting axons within optic nerve cross-sections 4 weeks after laser treatment.

Results. After intravitreal transplantation, MSCs survived for at least 5 weeks. Cells were found mainly in the vitreous cavity, though a small proportion of discrete cells migrated into the host retina. Intravitreal MSC transplantation resulted in a statistically significant increase in overall RGC axon survival and a significant decrease in the rate of RGC axon loss normalized to cumulative intraocular pressure exposure. After intravenous transplantation, MSCs did not migrate to the injured eye. Intravenous transplantation had no effect on optic nerve damage.

Conclusions. Local, but not systemic, transplantation of MSCs was neuroprotective in a rat glaucoma model. Autologous intravitreal transplantation of MSCs should be investigated further as a potential neuroprotective therapy for glaucoma. (*Invest Ophthalmol Vis Sci.* 2010;51:2051–2059) DOI:10.1167/iovs.09-4509

Glaucoma is a chronic, neurodegenerative disease for which current treatments slow progression but cannot always halt visual deterioration. Current therapies work by reducing intraocular pressure (IOP), the most important risk factor for the onset and progression of the disease. However, progressive visual loss continues in a proportion of patients despite maximally tolerated ocular hypotensive therapy. Thus, the development of novel therapies that protect RGCs from degeneration is crucial.

Research has implicated impaired RGC retrograde transport of neurotrophic factors (NTFs) as a contributory mechanism in glaucoma.[1–5] Transport blockade occurs at the level of the optic nerve head and can be correlated with elevated IOP in rodent, canine, and primate models of the disease.[1–5] Accordingly, it has been demonstrated that supplementing RGCs with NTFs, either by direct application of protein[6,7] or by transduction of endogenous cells,[8–10] slows the loss of these neurons in models of optic nerve disease and glaucoma. However, long-term maintenance of the NTF supply has proved problematic.

Recently, transplanted mesenchymal stem/stromal cells (MSCs) have demonstrated significant neuroprotection in several central nervous system degenerative models.[11–14] MSCs can be isolated from a variety of tissues, including the adult bone marrow, and have the potential to differentiate into adipocytes, osteocytes, and chondrocytes. It has been suggested that MSCs may transdifferentiate into neural cells, although this remains controversial.[15] However, MSCs have a key characteristic that makes them attractive candidates for cell-based neuroprotective therapies. They produce and secrete a battery of both NTFs and anti-inflammatory cytokines in situ after transplantation. Secretion of these factors appears to confer strong neuroprotective effects in models of amyotrophic lateral sclerosis (ALS),[16,17] multiple sclerosis (MS),[18,19] Parkinson's disease (PD),[20,21] ischemia,[22–24] and spinal cord injury.[25,26] Successful cell therapy protocols have included local and systemic transplantation. To our knowledge, systemic stem cell delivery has not yet been investigated as a treatment for glaucoma. In addition, MSCs are attractive because they may provide an autologous approach to cell-based therapies.

Our aim was to determine whether transplantation of MSCs could be a viable therapeutic approach to treating glaucoma. We isolated and syngeneically transplanted MSCs in two different rat breeds: inbred Lewis and outbred Sprague-Dawley (SD). We found that MSCs delivered locally produced a robust neuroprotective effect but that, contrary to its effect in other neurodegenerative conditions, systemic administration was not beneficial in glaucoma.

Methods

Animals

Adult (8- to 12-week-old) male wild-type (WT) SD rats, transgenic green fluorescent protein (GFP)-expressing SD rats, and WT Lewis rats were housed in light- and temperature-controlled conditions. All animals were immunocompetent. All procedures were carried out in accordance with UK Home Office regulations for the care and use of laboratory animals, the UK Animals (Scientific Procedures) Act (1986),

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Table 1. Summary of Experimental Groups

Recipient Strain	Transplant	Route of Administration	*n*
WT Lewis	Dead WT Lewis MSC	Intravitreal	10
WT Lewis	Live WT Lewis MSC	Intravitreal	10
WT SD	Dead GFP$^+$ SD MSC	Intravitreal	9
WT SD	Live GFP$^+$ SD MSC	Intravitreal	10
WT SD	PBS	Intravenous	8
WT SD	Dead GFP$^+$ SD MSC	Intravenous	8
WT SD	Live GFP$^+$ SD MSC	Intravenous	9

Three separate experiments were carried out using the groups described above. WT Lewis or SD rats were used as recipients, and WT Lewis or GFP-expressing SD rats were used to derive donor MSCs for transplantation. PBS, 0.1 M phosphate-buffered saline; *n,* number of animals per group.

and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. For the purpose of analysis, animals undergoing laser-induced ocular hypertension (OHT) and transplantation experiments were divided into seven experimental groups, as described in Table 1.

Mesenchymal Stem Cells

MSCs were isolated from the femoral bone marrow of adult GFP-expressing SD rats and WT Lewis rats, as previously described.[27] Briefly, bone marrow was aspirated from the femurs and seeded into plastic culture flasks at a density of 5×10^5 cells/cm^2 in DMEM (1 g/L glucose) containing 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL; all from Invitrogen Inc., Carlsbad, CA). After 48 hours, nonadherent cells were removed by complete media exchange. Cells were grown until approximately 80% confluent and then were passaged as necessary. Previously published assays characterizing MSCs isolated using the same technique have confirmed the expression of CD90 and CD44 but not of CD34 or CD45.[27] Cells used in the present experiments were from passages 4 to 12.

MSC identity was verified at passages 3 to 5 and at passages 11 to 13 by inducing osteogenesis and adipogenesis, as described previously.[27] Briefly, osteogenesis was induced by supplementing the culture media with dexamethasone (0.1 μM), glycerophosphate (10 mM), and ascorbic acid (50 μM), whereas adipogenesis was triggered by supplementing the culture media with 1-methyl-3-isobutylxanthine (0.5 mM), dexamethasone (1 μM), insulin (10 μg/mL), and indomethacin (100 μM; all from Sigma-Aldrich, St. Louis, MO). Oil red O or alizarin red S staining was used to confirm differentiation into adipocytes and osteocytes, respectively.

MSC immunocytochemistry was carried out after fixation of cells with 4% paraformaldehyde (PFA) for 30 minutes at room temperature. Cells were simultaneously blocked and permeabilized with 0.2% Triton in 0.1 M phosphate-buffered saline (PBS) containing 5% goat serum for 1 hour at room temperature, incubated with primary antibodies diluted in blocking solution overnight at 4°C, and then incubated with appropriate fluorescent secondary antibodies (all diluted 1:1000; all from Invitrogen Inc.) in blocking solution for 1 hour at room temperature. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen Inc.). Primary antibodies raised against the following antigens were used: fibronectin (1:100; Abcam Inc., Cambridge, UK), vimentin (1:1000; Millipore, Billerica, MA), laminin (1:100; Sigma-Aldrich), nestin (1:200; Millipore), collagen IV (1:100; Abcam Inc.), ED1 (CD68; 1:100; Millipore), and CD11b (OX-42 clone; 1:100; Millipore).

Laser-Induced Ocular Hypertension

Ocular hypertension was induced using a modification of the method developed by Levkovitch-Verbin et al.[28] Briefly, rats were anesthetized with ketamine (50 mg/kg) and xylazine (10 mg/kg) injected intraperitoneally and were placed in front of a slit-lamp equipped with a 532-nm diode laser that delivered 0.7-W pulses for 0.6 seconds. Fifty to sixty laser pulses (50-μM diameter) were directed to the trabecular meshwork 360° around the circumference of the aqueous outflow area of the left eye. Animals were treated twice, 1 week apart. Contralateral fellow eyes served as untreated controls.

IOP was measured bilaterally under anesthesia before and 24 hours after each laser treatment and then weekly thereafter using a rebound tonometer (TonoLab; Tiolat Oy, Helsinki, Finland). Tonometry was performed within 5 minutes of anesthesia onset and always between the hours of 9:00 am and 11:00 am. Cumulative IOP exposure for each eye was calculated as the integral of the IOP over the 4-week period beginning with the first laser treatment.

Cell Transplantation

One week before the induction of ocular hypertension, 3×10^4 live MSCs or 3×10^4 dead MSCs (killed by incubation at 80°C for 30 minutes) in 3 μL sterile PBS were transplanted syngeneically into the vitreous cavity of the left eye under isoflurane anesthesia. Local anesthetic was also applied topically to the cornea. Cells were injected through the superior nasal retina using a 30-gauge needle on a 5-μL glass Hamilton syringe. Care was taken to ensure that the lens was not damaged and that the retinal blood supply was not affected. In a separate group, 5×10^6 live MSCs, 5×10^6 dead MSCs, or sterile equivalent volume (1 mL) PBS was injected intravenously through the tail vein immediately after the induction of ocular hypertension. Experimental groups are detailed in Table 1.

Tissue Preparation

Four weeks after the onset of ocular hypertension, animals were perfused transcardially with 4% PFA under terminal anesthesia. Eyes were enucleated, and the anterior segment including the lens was removed. Posterior eyecups were postfixed by immersion in 4% PFA for 24 hours at 4°C, cryoprotected in 30% sucrose for 24 hours at 4°C, and embedded in optimal cutting temperature compound (OCT; Raymond A. Lamb UK, Eastbourne, UK). Eyecups were cryosectioned at 40 μm directly onto microscope slides (Superfrost Plus; VWR International Ltd, Lutterworth, UK). Immunohistochemistry was carried out by simultaneously blocking and permeabilizing sections with 0.2% Triton in PBS containing 5% goat serum (Invitrogen Inc.) for 1 hour at room temperature, incubating with primary antibodies diluted in blocking solution overnight at 4°C, and incubating with appropriate fluorescent secondary antibodies (all diluted 1:1000; all from Invitrogen Inc.) in blocking solution for 3 hours at room temperature. Nuclei were counterstained with DAPI (Invitrogen Inc.). Primary antibodies raised against the following antigens were used: GFP (1:5000; Millipore), glial fibrillary acidic protein (GFAP; 1:500; Dako UK Ltd., Ely, UK), nestin (1:200; Millipore), ED1 (CD68; 1:500; Millipore), laminin (1:200; Sigma-Aldrich), and CD11b (OX-42 clone; 1:500; Millipore). Sections were visualized on a laser scanning confocal microscope (TCS-SPE; Leica Inc., Wetzlar, Germany).

For assessment of RGC axonal loss, optic nerves were immersed in 4% PFA/5% glutaraldehyde in phosphate buffer for 7 days at 4°C, postfixed in 1% osmium tetroxide for 3 hours, dehydrated, and embedded in Araldite resin for semithin sectioning. Semithin (1-μm) transverse sections were cut from the nerve 2 to 3 mm distal to the globe, dried onto slides, and stained with 1% toluidine blue.

Retinal Ganglion Cell Axon Quantification

Loss of RGC axons in the optic nerves of glaucomatous eyes was assessed using a modification of an established semiquantitative optic nerve grading scheme, as we have described in detail previously.[29] Briefly, zones of apparently homogenous damage were identified under light microscopy (100× magnification), and the percentage contribution of each zone to total ON cross-sectional area was determined (examples are provided in Supplementary Figs. S1–S3, http://www.iovs.org/cgi/content/full/51/4/2051/DC1). A representative photograph within each zone was captured at 630× magnification (representing

0.004-mm^2 total optic nerve area), and the number of axons within each sample image was quantified using ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). The number of unique zones of damage within optic nerve cross-sections ranged from 1 to 3; hence, the total area of optic nerve assessed ranged from 0.004 to 0.012 mm^2 or approximately 2% to 6% of the optic nerve cross-sectional area. The number of axons within each damage zone was compared with the count obtained from a sample image of the uninjured companion eye to estimate the percentage of axonal survival. A weighted average calculation was then used to estimate the percentage of surviving axons in the total ON for each animal based on the proportion of the total optic nerve cross-sectional area that each damage zone represented.

In a separate, prior study, a comparison of the percentage RGC axon survival obtained using the present method with full axon counts of 100% of the optic nerve cross-sectional area (the gold standard) in six adult male Wistar rats with laser-induced ocular hypertension and a range of optic nerve damage from mild to severe yielded a very strong correlation (slope = 0.94 ± 0.09; *y*-intercept = 1.94 ± 4.756; R^2 = 0.96). In contrast, the comparison of an alternative, commonly used method that relies on random sampling of a proportion of the optic nerve cross-sectional area[8,30,31] (e.g., 10 fields of view corresponding to approximately 20% of the cross-sectional optic nerve area) in the same group of animals yielded a less robust correlation (slope = 0.91 ± 0.30; *y*-intercept = 9.20 ± 15.11; R^2 = 0.69; Marina and Martin, manuscript in preparation). Best-fit linear regressions for these two correlations are presented in Supplementary Figure S4. Thus, it appears that the present method can provide an accurate representation of RGC axonal survival throughout the entire optic nerve with less risk of missing focal areas of significant damage

Statistical Analysis

IOP, cumulative IOP exposure for each eye, RGC axon survival, and RGC axon loss normalized to cumulative IOP exposure were compared among animals that received live and dead intravitreal MSC transplants using unpaired, two-tailed Student's *t*-tests. The same parameters were compared among animals receiving PBS, dead MSCs, or live MSCs intravenously using one-way analyses of variance (ANOVA). Data are expressed as mean ± standard error of the mean (SEM).

Results

Characterization of MSCs In Vitro

MSCs were isolated from transgenic GFP-SD and WT Lewis rats. MSCs exhibited typical fibroblast morphology (Fig. 1A). After four passages of expansion and purification, both lines of cells were assayed for multilineage differentiation potential. After induction, MSCs were able to differentiate into mature osteocytes, as confirmed by alizarin red S staining of calcium deposits (Fig. 1B), and mature adipocytes, as confirmed by oil red O staining of intracellular lipid vacuoles (Fig. 1C). Immunofluorescence analysis demonstrated that >99% of the cells in culture expressed common markers of mesodermal lineage, such as fibronectin, laminin, collagen IV, and vimentin (Figs. 1D–F, 1H, Supplementary Figs. S5A–S5C). In addition, approximately one-third of MSCs expressed high levels of nestin, an intermediate filament sometimes associated with neural progenitor cells (Fig. 1G, Supplementary Fig. S5D). MSCs did not express CD11b (Fig. 1I, Supplementary Fig. S5E) or ED1 (Supplementary Fig. S5F), markers of monocyte lineage, indicating that the cultures were free of hematopoietic stem cell contamination.

Glaucoma Model

Ocular hypertension was induced by laser photocoagulation of the trabecular meshwork. The IOP profiles for both eyes of animals within each experimental group are shown in Figures 2A, 2C, 2E. Compared with contralateral control eyes, in which all seven groups had mean IOPs between 10.1 ± 0.4 mm Hg and 12.4 ± 0.5 mm Hg at all time points, laser treatment resulted in a transient elevation of intraocular pressure to between 22.6 ± 2.3 mm Hg (in the animals that received live Lewis MSCs intravitreally) and 45.8 ± 3.0 mm Hg (in the animals that received dead SD MSCs intravitreally; Figs. 2A, 2C, 2E). No significant difference in IOP profile was observed in ocular hypertensive eyes that received live or dead intravitreal Lewis transplants (Fig. 2A) or those that received intravenous transplants (Fig. 2E). However, rats that received intravitreal dead SD MSCs had significantly ($P \leq 0.05$) higher mean IOPs on the day after each laser treatment and 1 week after the second laser treatment than rats that received live SD MSCs (Fig. 2C).

To compare cumulative IOP exposure from treatment onset, integral IOP was calculated over the course of the 4

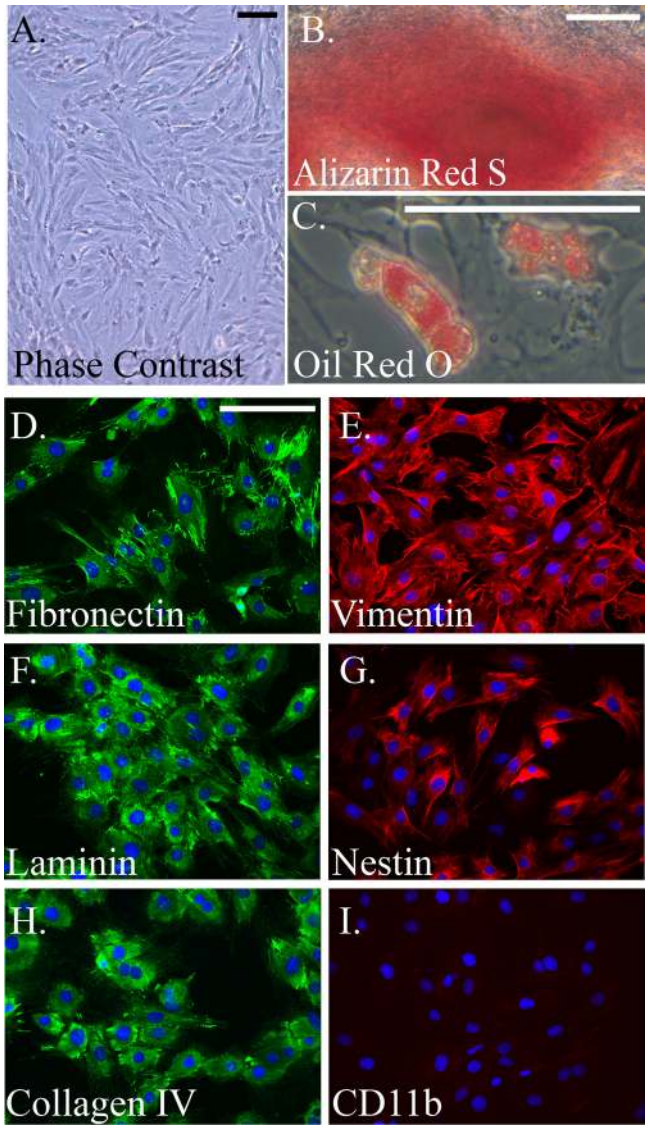

Figure 1. MSC characterization. Cultured MSCs exhibited typical fibroblast morphology under phase contrast (**A**). MSCs differentiated into osteocytes, as indicated by positive staining for calcium deposits with alizarin red S (**B**) and adipocytes, as indicated by positive staining for lipid vacuoles with oil red O (**C**). Immunocytochemistry revealed that undifferentiated MSCs expressed fibronectin (**D**), vimentin (**E**), laminin (**F**), and collagen IV (**H**). Some MSCs expressed nestin (**G**). MSCs did not express the monocyte marker CD11b (**I**). WT (GFP$^-$) Lewis MSCs are shown. Scale bars, 100 μm (**A–D**). DAPI, *blue* (**D–I**).

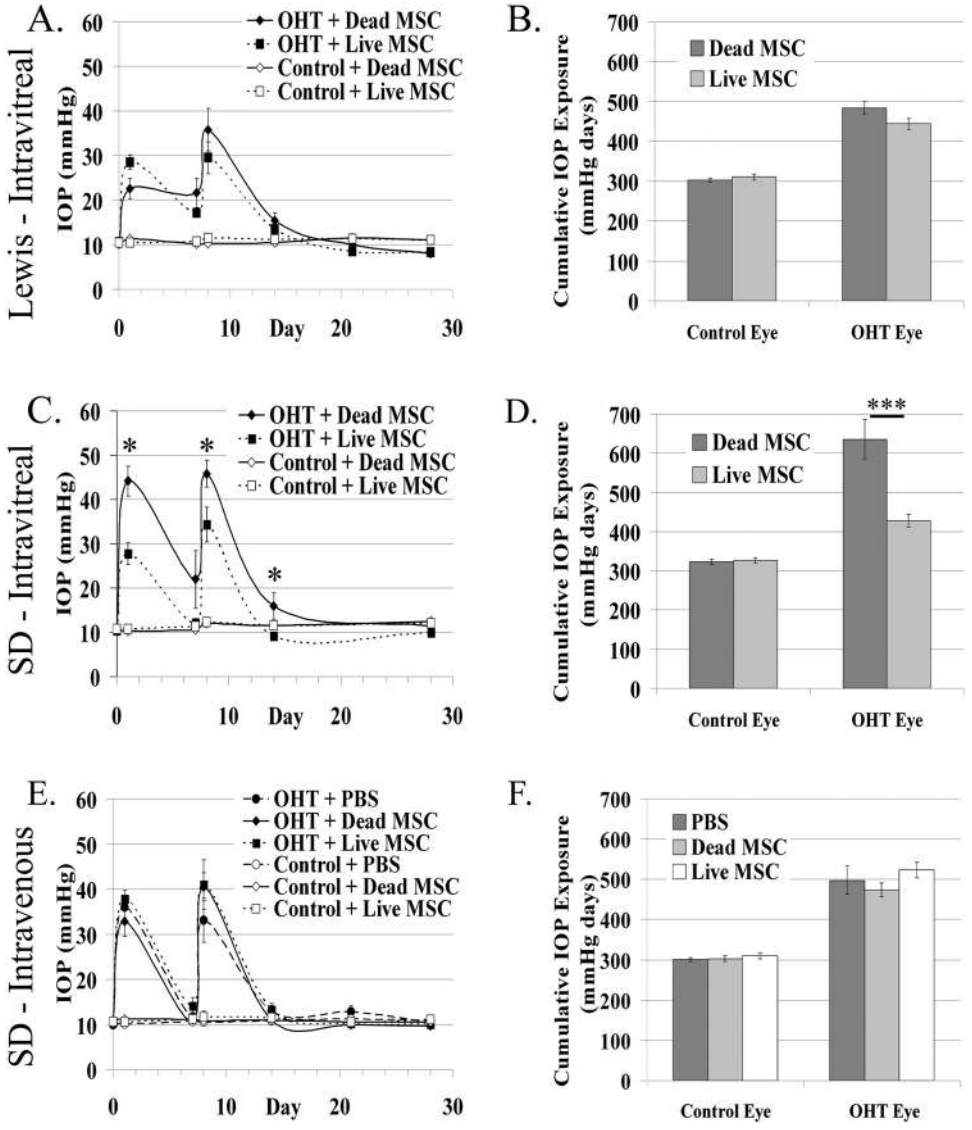

FIGURE 2. IOP analysis. Laser photocoagulation of the trabecular meshwork to induce OHT took place on days 1 and 8, and each treatment was followed by an elevation of IOP (**A**, **C**, **E**). Cumulative IOP exposure in untreated control eyes and eyes that received laser-induced OHT was calculated as the integral of IOP over the 4-week experimental period (**B**, **D**, **F**). Error bars represent SEM. $^{*}P \leq 0.05$ by unpaired *t*-test comparing OHT groups at the indicated time point; $^{***}P < 0.001$ by unpaired *t*-test. The number of animals in each group can be found in Table 1.

experimental weeks. Untreated eyes exhibited consistent cumulative IOP exposures that averaged 311 ± 3 mm Hg days ($N = 64$) with no significant differences between groups (Figs. 2B, 2D, 2F). Laser treatment increased cumulative IOP exposure to between 428 ± 16 mm Hg days (in the animals that received live SD MSCs intravitreally) and 636 ± 51 mm Hg days (in the animals that received dead SD MSCs intravitreally; Figs. 2B, 2D, 2F). No difference in the cumulative IOP for the OHT eye was observed between animals that received live versus dead intravitreal Lewis transplants (Fig 2B) or those that received intravenous transplants (Fig. 2F). However, rats that received intravitreal dead SD MSCs had significantly ($P \leq 0.001$) higher cumulative IOP exposure in the experimental eye than rats that received live MSCs (Fig. 2D).

Localization of Transplanted MSCs

Experiments using syngeneic transplantation of GFP^+ cells into WT SD rats allowed tracking of the grafted cells 5 weeks after transplantation (4 weeks after the onset of ocular hypertension). Fluorescence microscopy demonstrated that intravitreally transplanted MSCs survived well in the posterior segment of ocular hypertensive eyes. The vast majority of grafted cells remained as a bolus within the vitreous cavity and sometimes attached to the posterior lens capsule, which was identified by its strong expression of laminin (Figs. 3A, 3B, Supplementary Figs. S6A, S6C). In addition, GFP^+ MSCs were found to colocalize with laminin, indicating that they continued to express this mesodermal marker in vivo (Fig. 3B, Supplementary Fig. S6C). $ED1^+$ (Fig. 3B, Supplementary Figs. S6A, S6C) and $CD11b^+$ (Supplementary Fig. S6B) inflammatory cells were frequently found near, or infiltrating, the graft bolus. A small number of MSCs migrated into the host tissue (Figs. 3C–H). These cells clearly localized underneath the inner limiting membrane (detected with a laminin antibody) and within the nerve fiber layer or retinal ganglion cell layer of the host retina. Immunodetection of the macrophage/monocyte marker ED1 was used to discriminate grafted MSCs from inflammatory cells that appeared GFP^+ because of phagocytosis or autofluorescence (Figs. 3E–H). In addition, MSCs preferentially migrated to perivascular sites (Figs. 3C, 3E).

Inflammatory cells (microglia/monocytes/macrophages immunoreactive for ED1 and monocytes/granulocytes/macrophages/NK cells immunoreactive for CD11b) were found within the vitreous cavity infiltrating the live MSC grafts (Fig. 3B, Supplementary Figs. S6A–S6C) but were not detected within the vitreous cavity distal to MSCs. Furthermore, inflammatory cells were not observed in the vitreous cavity of eyes that received dead MSC grafts at the 5 week time point assessed, though it is likely they were present earlier to clear cell debris. Within the retina, there was some evidence of micro-

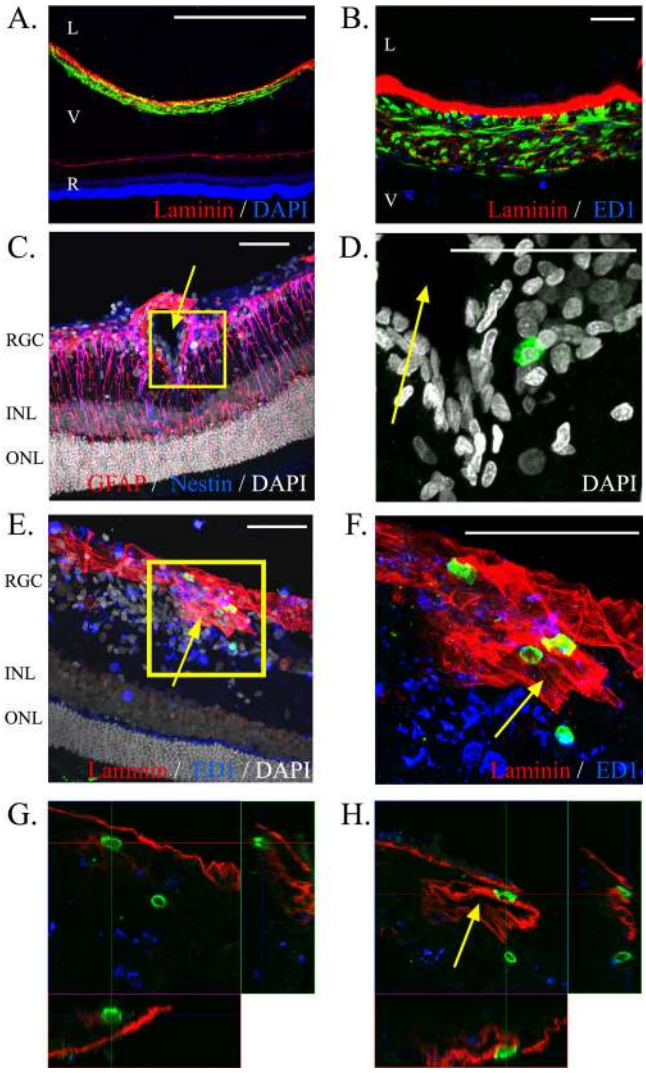

FIGURE 3. MSC localization after intravitreal transplantation. Immunohistochemistry revealed that GFP$^+$ mesenchymal stem cells (MSCs, *green*) survived in the posterior eyecup up to 5 weeks after transplantation. (**A**, **B**) Most MSCs were found as a bolus within the vitreous, often adherent to the posterior lens capsule (strongly immunoreactive for laminin; *red*; **A**, low magnification; **B**, high magnification). (**C–H**) In rare cases, discrete MSCs migrated to the host retina and were almost exclusively localized in the nerve fiber layer or ganglion cell layer. (**D**, **F**) Higher magnification of the areas within the *yellow squares* in (**C**) and (**E**), respectively. (**G**, **H**) Single orthogonal sections from the maximal projection confocal z-stack depicted in (**F**). L, lens; V, vitreous; R, retina; RGC, retinal ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. *Yellow arrows*: blood vessels. Scale bars: 500 μm (**A**); 50 μm (**B–F**).

glial activation in response to ocular hypertension, but no obvious differences were observed between eyes that received live or dead intravitreal MSC grafts (S6D, E).

Migration of MSCs to the retina was also examined after intravenous transplantation in ocular hypertensive animals; however, extensive immunohistochemical analysis failed to detect any transplanted cells in the posterior eyecup. Discrete cells with a fluorescence profile similar to that of GFP were observed within the retina and vitreous but uniformly expressed the macrophage/monocyte/microglial marker ED1 (Fig. 4). These cells were identified in animals that received intravenous transplantation of both live and dead MSCs and those that received only PBS. We were unable to identify differences in GFP labeling between animals that received transplants containing GFP$^+$ cells compared with PBS controls.

RGC Survival after MSC Transplantation in Glaucoma

Quantification of optic nerve damage demonstrated that intravitreal MSC transplantation was significantly neuroprotective in experimental glaucoma. Lewis rats that received live MSC grafts exhibited 92.1% ± 2.4% RGC axon survival 4 weeks after the onset of ocular hypertension compared with 74.7% ± 5.5% for animals that received dead MSCs ($P < 0.01$; Fig. 5A). Similarly, SD rats that received intravitreal live MSCs had 89.3% ± 3.5% RGC axon survival compared with 61.7% ± 9.1% for animals that received dead MSCs ($P < 0.01$; Fig. 5C). In addition, a more robust analysis of neuroprotection was carried out by normalizing RGC axon loss to cumulative IOP exposure because individual injury exposure was somewhat variable. This analysis also demonstrated that intravitreal MSC transplantation was neuroprotective: Lewis rats that received live MSC grafts lost RGC axons at a rate of 0.018% ± 0.006%/mm Hg per day compared with 0.051% ± 0.011%/mm Hg per day for animals that received dead MSCs ($P = 0.01$; Fig. 5B). Similarly, SD rats that received live MSC grafts exhibited RGC axon loss at a rate of 0.023% ± 0.008%/mm Hg per day compared with 0.058% ± 0.013%/mm Hg per day for animals that received dead MSCs ($P < 0.05$; Fig. 5D).

A similar analysis was conducted in ocular hypertensive animals that received intravenous injections of saline, dead MSCs, or live MSCs. Analysis of uncorrected RGC axon survival and RGC axon loss normalized to cumulative IOP exposure in the hypertensive eye revealed no significant difference in optic nerve damage among the three groups (Fig. 6).

DISCUSSION

The present study demonstrated that intravitreal transplantation of MSCs conferred neuroprotection in a rat model of glaucoma. This supports our previous study that showed intravitreal delivery of neural stem cells can protect RGCs from glaucomatous death.[29] However, difficulties associated with obtaining and using neural stem cells for cell-based therapies are likely to impair their translation to the clinic. Other cell sources, such as embryonic or fetal stem cells, have also demonstrated retinal neuroprotective qualities,[32–34] but their use is limited by rejection and ethical issues. In contrast, MSCs may be obtained from individual patients permitting autologous transplantation, thereby avoiding such hurdles and facilitating their use for neuroprotective strategies in the treatment of degenerative conditions. Our successful demonstration of MSC-mediated optic nerve protection in a model of glaucoma suggests that these cells could be transplanted to slow glaucomatous disease progression in patients by directly protecting surviving RGCs and their axons in an IOP-independent manner.

The present study assessed optic nerve damage, quantified by RGC axon loss, as the primary outcome measure for MSC-mediated neuroprotection. It should be noted that the survival of RGC cell bodies within the retina was not directly investigated. However, given that it has been noted previously that RGC axonal degeneration can occur with relative sparing of RGC bodies[35] and given that RGC soma protection in the context of optic nerve degeneration would likely not provide functional benefit in glaucoma, we feel that the present analysis uses a reasonable end point for effective neuroprotection. In addition, the present study demonstrated significant protection of the optic nerve after MSC transplantation, as assessed by both overall RGC axon survival and RGC axon survival normalized to cumulative IOP exposure. Our study relied on single-peak IOP measurements made 24 hours after laser treatment to

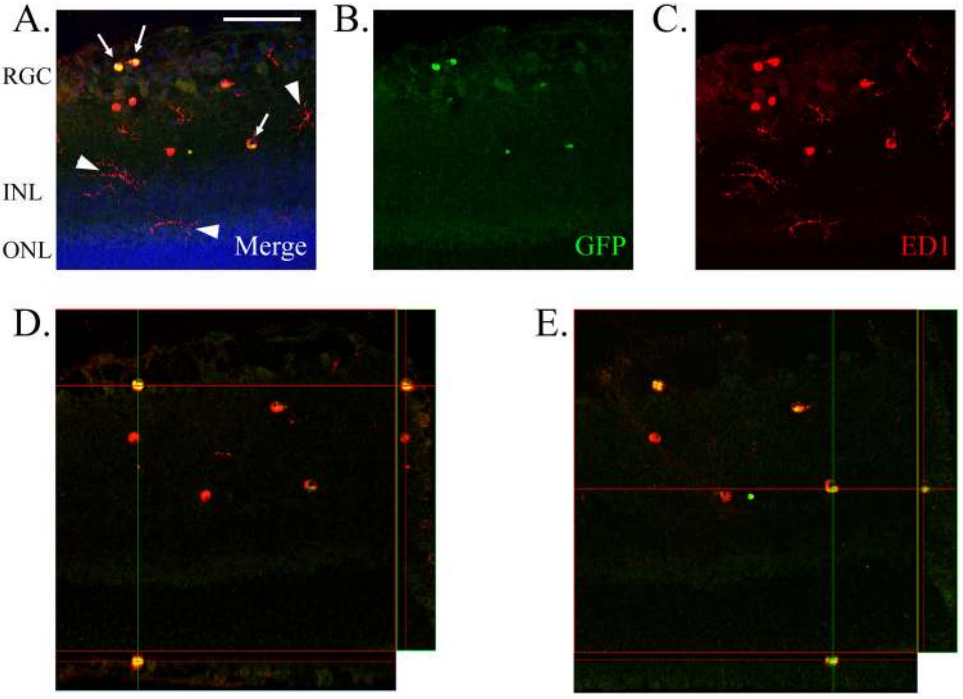

FIGURE 4. Absence of $GFP^+/ED1^-$ MSCs in the eye after intravenous transplantation. Confocal analysis revealed that ED1 expression (macrophage/monocyte marker) colocalized with GFP^+ cells within the posterior eyecups of animals that received intravenous GFP^+ MSC transplants, indicating erroneous GFP labeling of infiltrating macrophages or microglial cells rather than engrafted MSCs (**A–C**). (**A**, *arrows*) $GFP^+/ED1^+$ macrophages/microglia with activated morphology. (**A**, *arrowheads*) $GFP^-/ED1^+$ microglia with a resting, ramified morphology. (**D**, **E**) Orthogonal sections from the maximal projection confocal z-stack depicted in (**A**) to (**C**). Scale bar, 50 μm.

extrapolate cumulative IOP exposure because of a limit on multiple anesthesia contained in our license from the UK Home Office. Although cumulative IOP exposure calculated using a similar number of time points has previously been shown to correlate strongly with percentage RGC axon loss in the same model of laser-induced ocular hypertension ($R^2 = 0.40$; $P < 0.0001$),[28] the limitations of this measure should be recognized. Investigators using this model should consider measuring IOP more frequently, for example in awake animals, to obtain a more precise cumulative IOP exposure measurement.

MSCs were observed to survive well within the recipient eye after intravitreal transplantation, at least for the 5-week study duration, despite no form of immunosuppression. Furthermore, no difference in survival after transplantation into either inbred or outbred rat breeds was found. Infiltrating inflammatory cells were identified juxtaposed to engrafted MSCs, but because graft survival was robust, it was unclear whether this indicated graft rejection or whether MSCs were interacting with inflammatory cells in some other capacity. Nonetheless, long-term graft survival augurs well for the development of cell-based neuroprotective therapies because sustained benefit from a single treatment would be ideal.

Local delivery of MSCs has also proved neuroprotective for other models of retinal degeneration, suggestive of a broadly effective mechanism. In related work, Yu et al.[36] found that intravitreal MSC transplantation was neuroprotective after episcleral vein ligature, which can cause moderate ocular hypertension and RGC loss. As Morrison et al.[37] have noted, however, IOP elevation in this model may involve ocular venous congestion rather than reduction of aqueous outflow and, therefore, may not induce the same type of injury to RGCs and the optic nerve that occurs in glaucoma. Interestingly, distal MSC transplantation after RGC axotomy at the level of the lateral geniculate nucleus is also reported to protect RGC bodies from degeneration.[38] In addition, local delivery of MSCs has reduced retinal neurodegeneration in other pathologic models, such as that after ischemia/reperfusion[23] and in models of photoreceptor degeneration.[39,40] Our results clearly support these earlier findings. However, the present study is the first to demonstrate a robust neuroprotective effect in a model that involves RGC insult through ocular hypertension induced by aqueous outflow obstruction. Moreover, we demonstrated that intravenous MSC delivery is unlikely to prove efficacious as a novel neuroprotective therapy in glaucoma.

The most likely mechanism underlying the neuroprotective effect described by the present study is the secretion of NTFs by the grafted MSCs. It is well established that MSCs isolated from mice, rats, and humans secrete brain-derived neurotrophic factor, glial-derived neurotrophic factor, nerve growth factor, and ciliary neurotrophic factor, among other neurotrophic factors. Indeed, NTF transcription, translation, and secretion by MSCs has been demonstrated using methodologies such as quantitative and semiquantitative RT-PCR,[23,36,41,42] immunocytochemistry,[23,36,42] immunoblot analysis,[23] and ELISA.[23,36,38–44] It should be noted, however, that most data suggesting that MSCs secrete NTFs have been obtained using in vitro culture systems; whether this phenomenon occurs at similar levels in vivo is as yet unknown. Nonetheless, these cells have been shown to support the growth and survival of neuronal cell cultures and ameliorate neuronal cell loss in a variety of neurodegenerative diseases.[16–26] If proven, this mechanism of action may be particularly pertinent in glaucoma, in which reduced bioavailability of these proteins to RGCs appears to play a key role in the pathophysiology of the disease.[1–5]

In addition to providing NTFs, MSCs may also achieve neuroprotection in degenerative CNS pathologies by modulating the inflammatory immune response. A possible immunomodulatory neuroprotective mechanism has been described for neural stem cells transplanted into a neurodegenerative demyelination model of MS.[45] After intravenous delivery, neural stem cells migrated into the inflamed CNS, mediated by their expression of integrin and chemokine receptors, accumulated in perivascular sites, and reduced neuronal death. Once localized perivascularly, the neural stem cells appeared to trigger the apoptosis of CNS-infiltrating inflammatory cells, thereby protecting neurons from chronic degeneration. A similar mechanism of action has been proposed to contribute to MSC-mediated neuroprotection.[46,47] The possibility of treating localized neurodegenerative pathologies, such as glaucoma or stroke, or multifocal diseases, such as MS, with systemic stem cell ther-

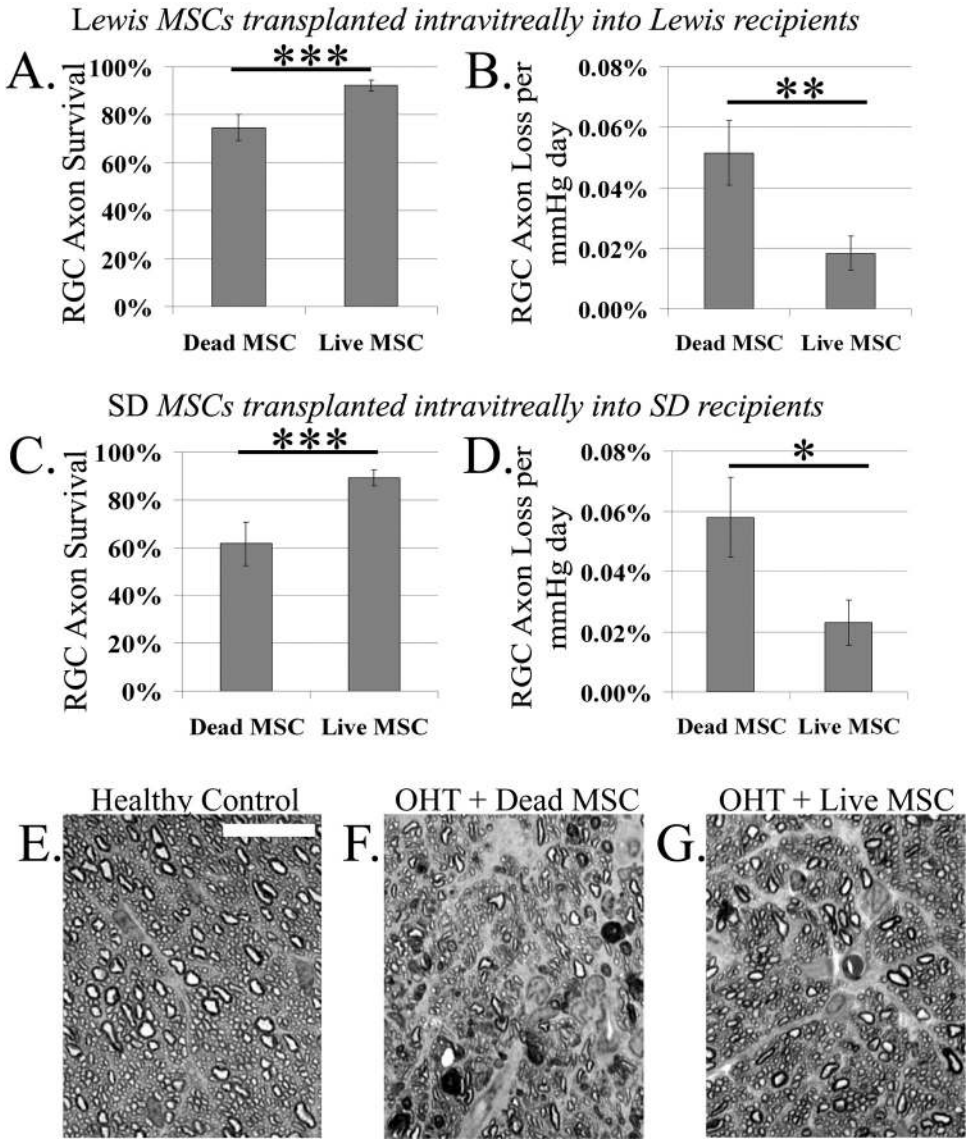

FIGURE 5. RGC axon survival after intravitreal MSC transplantation. (**A**, **B**) Results of experiments using Lewis MSCs and recipients (dead MSCs, $n = 10$; live MSCs, $n = 10$). (**C**, **D**) Results of experiments using SD MSCs and recipients (dead MSCs, $n = 9$; live MSCs, $n = 10$). (**A**, **C**) RGC axon survival was calculated as the percentage of surviving RGC axons in ocular hypertensive optic nerves compared with contralateral control tissue. (**B**, **D**) RGC axon loss normalized to cumulative IOP exposure was calculated as the percentage of RGC axon loss divided by the integral IOP exposure experienced by the ocular hypertensive eye over 4 weeks. (**E**) Representative micrographs of healthy control optic nerves, (**F**) ocular hypertensive optic nerves from animals that received dead MSC transplants, and (**G**) ocular hypertensive optic nerves from animals that received live MSC transplants. Error bars represent SEM. $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, $^{*}P \leq 0.05$ (unpaired *t*-tests). Scale bar, 20 μm.

apy is attractive because it avoids possible neural trauma caused by direct transplantation and could aid widespread delivery. Indeed, intravenous MSC transplantation has demonstrated robust neuroprotection in several models of CNS degeneration[48–52] and is approaching clinical translation. In contrast, our data indicate that intravenous delivery of MSCs in our glaucoma model cannot protect RGCs from chronic degeneration. Furthermore, in this model, MSCs failed to migrate to the injured retina from the vasculature, as has been reported for other CNS pathologies.[47] It is unclear why this CNS-infiltrating behavior could not be triggered after retinal injury; however, glaucomatous neurodegeneration may be significantly less inflammatory than the chronic pathology occurring in demyelinating or ischemic disease. As such, selective death of RGCs may not generate a chemoattractive signal sufficient to recruit blood-borne stem cells for effective neuroprotection.

Differences in inflammatory response and protective autoimmunity between rat breeds have been documented in the past.[53–59] Therefore, we investigated whether MSC-mediated RGC neuroprotection varied between two rat types, inbred Lewis and outbred SD rats. No difference in the ability of MSCs to reduce optic nerve damage, when delivered intravitreally, was observed between rat breeds. This is suggestive of a robust, common, neuroprotective mechanism unconnected to possible differences in inflammatory reaction. The only difference observed between breeds was a tendency for glaucomatous SD rats that received live MSCs to have lower IOP than glaucomatous SD rats that received dead MSCs. This difference is likely to have resulted from model variability, but we cannot rule out that the transplantation of dead MSCs augmented IOP elevation in SD rats. Conversely, it is conceivable that intraocular injection of live MSCs directly alleviated IOP elevation in SD rats. Intravitreal transplantation of stem cells (alive or dead) has not been reported to affect ocular hypertension, and this effect was not observed in our previous studies which used other stem cell types.[29,60] In addition, this effect was not observed in the glaucomatous Lewis rats which also received intravitreal MSC transplants. Nonetheless, MSC-mediated suppression of local inflammation or debris generated by MSCs cell death may have affected the hypertensive response normally achieved with this model of glaucoma; further investigation would be required to confirm this effect. Regardless, it is important to note that the neuroprotective effect of MSC transplantation was significant in these animals, even after correction of the difference in cumulative IOP exposure, and that a comparable neuroprotective effect was found in the Lewis rats which experienced similar cumulative IOP exposures.

Previously, we showed that intravitreal transplantation of dead cells does not affect RGC survival in the laser-induced model of ocular hypertension when compared with saline

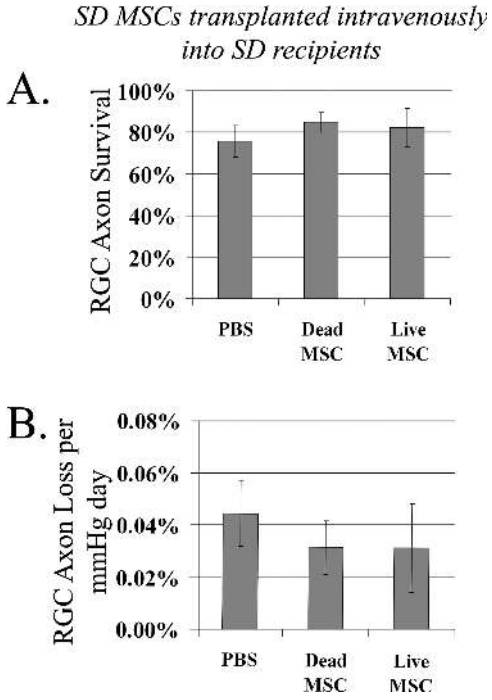

FIGURE 6. RGC axon survival after intravenous MSC transplantation. PBS ($n = 8$) or SD MSCs (dead MSCs, $n = 8$; live MSCs, $n = 9$) were injected intravenously into SD recipients. (**A**) RGC axon survival was calculated as the percentage of surviving RGC axons in ocular hypertensive optic nerves compared with contralateral control tissue. (**B**) RGC axon loss normalized to cumulative IOP exposure was calculated as the percentage of RGC axon loss divided by the integral IOP exposure experienced by the ocular hypertensive eye over 4 weeks. Error bars represent SEM. No significant differences between groups were found after ANOVA.

injection.[29] Similarly, the present study demonstrated that RGC survival after intravenous injection of either dead cells or PBS is equivalent in this model. Nonetheless, the use of dead MSCs as a negative control for intravitreal and intravenous transplantation is important as it more closely controls for any potential inflammatory responses to injection and delivers a vehicle of more similar molecular composition to live cell suspensions than saline without introducing live cells.

In this study we have demonstrated that the transplantation of MSCs can strongly protect the optic nerve from glaucomatous degeneration in an experimental model. This neuroprotective effect was repeatable and observed in both inbred and outbred rats. Although other stem cell types are also known to be neuroprotective, the use of MSCs for cell-based therapies is highly attractive because they may be used for autologous cell transplantation, thus avoiding the risk of rejection or the need for immunosuppressive agents. Further investigation is needed to determine the long-term efficacy of this treatment, including the survival of and sustained NTF secretion by the engrafted cells. In addition, safety must be carefully assessed to ensure that the graft will not be detrimental to visual function because the cells tend to remain in the vitreous cavity. Given that stem cells can retain proliferative capacity, research must ensure that grafted cells will not generate tumors. Even if the neuroprotective potential of MSC transplantation is confirmed in future human studies, adequate IOP control will remain essential to glaucoma treatment. Nonetheless, adjunctive neuroprotective therapies are much needed for clinical glaucoma management, and successful neuroprotection in this preclinical model suggests that intravitreal, autologous MSC transplantation should be investigated further as a potential future therapy for glaucoma.

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