Human Müller Stem Cell (MIO-M1) Transplantation in a Rat Model of Glaucoma: Survival, Differentiation, and Integration

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Purpose. Stem cell transplantation is a potential treatment strategy for neurodegenerative diseases such as glaucoma. The Müller stem cell line MIO-M1 can be differentiated to produce retinal neurons and glia. The survival, migration, differentiation, and integration of MIO-M1 cells were investigated in a rat model of glaucoma. The effect of modulating the retinal environment with either chondroitinase ABC or erythropoietin was also studied.

Methods. Intraocular pressure was chronically increased unilaterally by using a laser glaucoma model in adult rats. EGFP-transduced MIO-M1 cells were transplanted into the vitreous or subretinal space of glaucomatous or untreated eyes. Oral immune suppressants were administered to reduce xenograft rejection. Survival, migration, differentiation, and integration of grafted cells were assessed by immunohistochemistry.

Results. Transplanted cells survived for 2 to 3 weeks in vivo, although microglia/macrophage infiltration and a reduction in graft survival were seen by 4 weeks. Grafted cells displayed a migratory phenotype with an elongated bipolar shape often oriented toward the retina. Transplanted cells expressed markers such as PSA-NCAM, GFAP, and β-III-tubulin. The host retina was resistant to MIO-M1 migration, but modification of the local environment with erythropoietin or chondroitinase ABC facilitated retinal infiltration by MIO-M1 cells.

Conclusions. The results demonstrate that differentiating MIO-M1 cells within the glaucomatous eye produced cells that expressed neuronal and glial cell markers. The retina was relatively resistant to transplant integration, and long-term xenograft survival was limited. However, local modulation of the retinal environment enhanced the integration of MIO-M1 cells into the glaucomatous retina. (*Invest Ophthalmol Vis Sci.* 2008;49:3449–3456) DOI:10.1167/iovs.08-1770

Glaucoma is the leading cause of irreversible blindness in the world and one of the most common neurodegenerative diseases.[1] The condition involves characteristic changes at the optic nerve head and retinal ganglion cell (RGC) death, resulting in progressive visual field loss. Glaucoma shares many pathophysiological features common to other neurodegenerative diseases, including impairment of axonal transport,[2] oxidative stress,[3] and reactive glial changes.[4] A common risk factor for glaucoma is increased intraocular pressure (IOP) and current treatments focus on lowering and stabilizing the IOP to slow disease progression. However, some people with glaucoma continue to lose sight despite low IOP, and there is currently no cure or treatment to reverse glaucomatous visual field loss.

The isolation and characterization of stem cells from the adult neural retina,[5–8] and from other regions of the central nervous system (CNS), has raised the possibility of developing treatments for previously irreversible neurodegenerative diseases, such as glaucoma. Such treatments will most likely require the transplantation and subsequent integration of stem cells or their progenitors. Successful stem cell therapies could work in several different ways. For example, undifferentiated or immature cells could integrate and differentiate to replace lost neurons and directly repair neuronal circuitry.[9–14] Alternatively, engrafted cells could provide trophic support for remaining neurons to ameliorate disease progression.[15–19] However, if any such a therapeutic effect is to be achieved, it is essential that a suitable stem cell type be chosen for transplantation and that transplanted cells survive and integrate into the host tissue.

Intraocular transplantation of stem cells is a rapidly growing research field. Interestingly, the uninjured retina appears very resistant to the integration of new cells,[10,20] and tissue injury has been observed to facilitate the movement of transplanted cells into the retina.[21,22] A variety of stem cells have been tested for integration, differentiation, and functional outcome in an assortment of retinal injury models; however, the behavior of stem cells in the glaucomatous eye has yet to be studied. Research has generally focused on the replacement or protection of photoreceptors in models such as the Royal College of Surgeons (RCS) rat,[23–27] although models of ischemic retinal damage,[10,28] mechanical injury,[22] and selective RGC injury[29] have also been studied. Cell types transplanted include embryonic stem (ES) cells,[7,26,30,31] neural stem cells,[10,22,27,28] and mesenchymal stem cells.[24,25] In some models, engrafted cells have demonstrated an ability to integrate into the degenerating retina with immunohistochemical evidence of differentiation of stem cells into mature cell types.[22,27,28,30] Furthermore, transplantation of stem cells has demonstrated potent retinal neuroprotection and functional improvement.[7,12,23–26]

Recently, a spontaneously immortalized Müller cell line (MIO-M1) has been generated from the postmortem human neural retina.[6,32] MIO-M1 cells possess stem cell–like characteristics and appear to be derived from a population of Müller glia. Similar cells lines have now been established from several donors. MIO-M1 cells exhibit extensive proliferation in vitro and are capable of differentiating into a variety of retinal neuron types. In addition, it has been demonstrated that these cells can integrate into the immature or the dystrophic RCS rat retina and differentiate in vivo.[32] Furthermore, the use of

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adult-derived retinal cell lines avoids the ethical concerns associated with cells isolated from embryonic or fetal tissue. These attributes make the MIO-M1 cells, and related cell lines, attractive candidates for the development of novel therapies for retinal neurodegeneration. In the present study, we investigated the potential of MIO-M1 Müller stem cells for retinal integration and differentiation in an established rat model of glaucoma.[33]

METHODS

MIO-M1 Cell Culture

The human MIO-M1 cell line was established and characterized previously.[6,32] Briefly, this cell line was derived from postmortem human neural retina and engineered to express green fluorescent protein (EGFP-transduced) to facilitate tracking on transplantation.[32] The MIO-M1 line was initially purified for EGFP expression by fluorescence-activated cell sorting. Cells were used for transplantation at passages 45 to 47. The cells were maintained as an adherent cell line in 75 cm^2 tissue culture flasks in D-MEM (containing 4500 mg/L glucose, sodium pyruvate and stabilized L-glutamine [GlutaMAX; Invitrogen, Paisley, UK]) plus 10% vol/vol fetal bovine serum (EU approved, heat inactivated; Invitrogen) and penicillin/streptomycin (Invitrogen). When confluent, the cells were washed once with PBS, detached from the flask by treatment with trypsin (Invitrogen), washed with complete cell culture medium, and split 1:5 into fresh flasks. This passaging procedure was also used to harvest cells in preparation for transplantation.

Some cells were also exposed to extracellular matrix (ECM gel; Sigma-Aldrich, Gillingham, UK)-coated flasks plus recombinant basic fibroblast growth factor (bFGF; 20 ng/mL; Sigma-Aldrich) for 3 days before harvest for transplantation ($n = 20$).

Animals

All animal experiments were conducted in accordance with the U.K. Home Office regulations for the care and use of laboratory animals, the U.K. Animals (Scientific Procedures) Act (1986) and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Young adult (8 weeks) male Wistar rats (Charles River Laboratories or Harlan, UK) were used ($n = 102$). Animals had unrestricted access to food and water and were maintained on a 12-hour light–dark cycle.

Immunosuppression

Pilot experiments indicated that no immune suppression or administration of cyclosporine alone were insufficient for the prevention of total graft rejection. Triple therapies have been used successfully to prevent graft rejection,[34] and the use of inhibitors of purine/pyrimidine synthesis has been shown to reduce the steroid dosages necessary to inhibit rejection.[35] All animals received a cocktail of three immune suppressant drugs (all from W&J Dunlops Ltd., Veterinary Wholesalers, Dunnington, UK) in the drinking water: cyclosporine (20 mg/kg/d), azathioprine (2 mg/kg/d), and prednisolone (0.5 mg/kg/d). The water was flavored (5% vol/vol blackcurrant cordial) to encourage adequate consumption, and administration of drugs was started 2 days before induction of glaucoma. Serum levels of cyclosporine were measured in a random sample of animals at termination and the average concentration was 494.2 ± 75 μg/L (mean ± SEM; $n = 5$). Systemic administration of immune suppressants has been shown to modulate immune activity in the retina.[36]

Glaucoma Model and Transplantation

The animals were anesthetized by intraperitoneal injection of ketamine (50 mg/kg) plus xylazine (5 mg/kg), and local anesthetic drops were applied to the eye. Baseline IOP was measured with a rebound tonometer (TonoLab; Tiolat Oy, Helsinki, Finland) that was factory calibrated for use in rats. All IOPs were recorded with the rats under light ketamine/xylazine anesthesia. Unilateral ocular hypertension was induced in the left eye ($n = 98$) by external translimbal treatment of the aqueous outflow area with a 532-nm diode laser, as described previously.[33] Initial treatment was 50 to 60 spots of 50-μm diameter, 700-mW power, and 0.6-second duration. Laser treatment was repeated 1 week later if the difference in IOP between the left and right eyes was less than 10 mm Hg. Four control rats did not receive laser treatment but underwent all other procedures (immune suppression, IOP measurements, and transplantation). Fourteen rats received laser treatment and were killed 4 weeks later to assess retinal ganglion cell axonal loss and compliance with a glaucoma model, as established previously.[33] After baseline IOP measurement (day 0), IOPs were recorded on days 1, 7, and 8 and weekly thereafter. Intraocular transplantation of MIO-M1 cells (3×10^4 cells resuspended in 3 μL medium) was performed 3 weeks after the induction of glaucoma ($n = 84$). Cells were injected under direct observation either intravitreally, close to the RGC layer (30-gauge needle; $n = 46$), or subretinally (34-gauge needle; $n = 42$). The posterior segment of the eye was observed during the injection by using a binocular operating microscope and a glass coverslip coupled to the cornea with carbomer gel (Viscotears; Novartis Pharmaceuticals, Camberley, UK). Some animals also received a concurrent (added to cell suspension) intraocular injection of 10 mU/eye[37] chondroitinase ABC (from *Proteus vulgaris*; protease free; Seikagaku, Tokyo, Japan; $n = 17$) or 200 ng/eye[38] recombinant rat erythropoietin (EPO; R&D Systems Europe, Abingdon, UK; $n = 12$). The animals were killed 1, 2, 3, or 4 weeks after transplantation.

Tissue Processing

For immunohistochemical analysis, the animals were transcardially perfused under terminal anesthesia with 0.1 M PBS followed by 4% paraformaldehyde/0.1 M PBS (PFA). The eyes were enucleated and postfixed by immersion in 4% PFA, either as whole eyes or posterior eyecups (after the removal of anterior chamber and lens), for 2 hours. The tissue was washed with PBS, cryopreserved with 30% sucrose, and embedded in optimal cutting temperature (OCT; Raymond A. Lamb UK, Eastbourne, UK) compound for frozen sectioning. Sections were cut 40 μm thick on a cryostat and mounted on slides (Superfrost Plus; VWR International, Lutterworth, UK).

For assessment of ganglion cell axonal loss, the animals were transcardially perfused under terminal anesthesia with 0.1 M phosphate buffer (PB) followed by 4% paraformaldehyde/2.5% glutaraldehyde/0.1 M PB (PFA/Glut fixative). The eyes were carefully enucleated to avoid stretching of the optic nerve (ON). After the animals were decapitated and the overlying connective tissue and skull removed, the ONs were cut at the level of the optic chiasm and postfixed in PFA/Glut fixative at 4°C for 7 days. The ONs were postfixed in 1% osmium tetroxide for 3 hours, dehydrated, and embedded in araldite resin for semithin sectioning. Semithin (1 μm) sections were dried onto slides and counterstained with 1% toluidine blue.

Immunohistochemistry

The sections were initially washed with PBS and then blocked with PBS containing 0.2% Triton (PBS-T) plus 5% normal goat serum (NGS). All antibodies were diluted in the blocking solution. The sections were incubated in primary antibodies overnight at 4°C. After thorough washing with PBS, appropriate fluorescent goat secondary antibodies (Invitrogen) were applied to the sections for 3 hours at room temperature. The slides were washed, counterstained with DAPI, and coverslipped (FluorSave reagent; Calbiochem/Merck Chemicals, Beeston, UK). The following primary antibodies were used: mouse IgG anti-β-III-tubulin (1:2000; Promega, Southhampton, UK); mouse IgG1 anti-human nuclear antigen (1:500; Chemicon, Millipore, Watford, UK); mouse IgG2a anti-CD11b (OX-42; 1:500; Chemicon); mouse IgG1 anti-ED1 (CD68; 1:500; Chemicon); mouse IgG1 anti-nestin (1:200; Chemicon); rabbit polyclonal anti-GFAP (1:1000; DAKO, Ely, UK); mouse IgM anti-polysialic acid-neural adhesion molecule (PSA-NCAM; 1:1000; Chemicon); and mouse IgG anti-chondroitin sulfate "stub" (clone 1B5) antibody (1:250; Seikagaku, Tokyo, Japan).

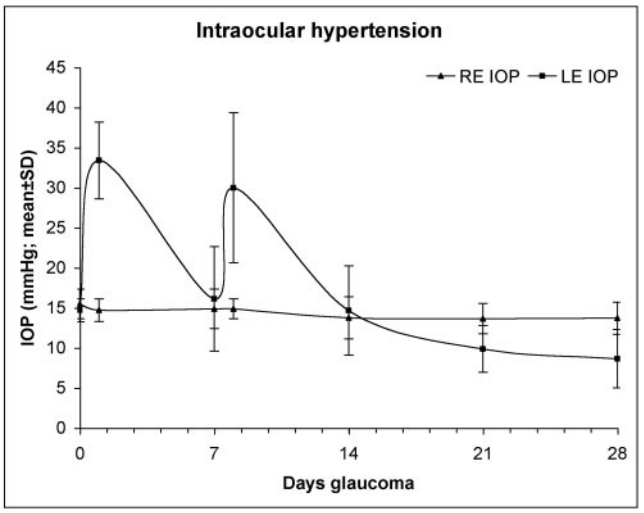

FIGURE 1. IOP profiles after the induction of ocular hypertension. Shown are IOP data from the left and right eyes of animals in which ocular hypertensive injury was induced in the left eye and retinal ganglion cell loss was quantified ($n = 14$; mean ± SD). Similar IOP profiles were obtained in all other animals subjected to ocular hypertension (Supplementary Fig. S1). RE, right eye; LE, left eye.

Quantification of Ganglion Cell Axonal Loss

Loss of ganglion cell axons in the optic nerves of glaucomatous eyes was assessed using a modification of a previously developed semiquantitative ON grading scheme.[39] This scheme divided the ON into zones of homogeneous damage, estimated the damage in each zone via visual grading, and obtained an average estimate of ON damage for each nerve. We used a similar method, but instead of estimating damage via visual grading, we used axon counts from representative images of each zone of damage to estimate total ON damage. To obtain the counts, we identified areas of approximately equal damage under light microscopy (100× magnification) and the contribution of each zone to total ON cross-sectional area was determined. A representative photograph was captured at 630× magnification within each zone, and the number of axons within each sample image was counted by using the particle-analysis/nucleus-counter plug-in (from the Wright Cell Imaging Facility; ImageJ plug-in bundle, University Health Network Research, Canada; available at http://www.uhnresearch.ca/facilities/wcif/fdownload.html) to the image-analysis software ImageJ (developed by Wayne Rasband, National Institutes of Health; available at http://rsb.info.nih.gov/ij/index.html). The number of axons within each damaged zone was compared to the count obtained from a sample image of the uninjured companion eye to estimate the percentage of axonal survival. A weighted average calculation (i.e., sum of the products of the mean percentage of surviving axons in each zone and the area of the ON occupied by that zone as per a previously established method[39]) was then used to estimate the percentage of surviving axons in the total ON.

RESULTS

Induction of Glaucoma Model and RGC Injury

Induction of ocular hypertension and the resultant ON damage was achieved as described previously by using this experimental model.[33] IOP was elevated in all laser-treated eyes (Fig. 1 and Supplementary Fig. S1; Supplementary Figures are online at http://www.iovs.org/cgi/content/full/49/8/3449/DC1). In animals with induced ocular hypertension alone, later used for assessment of ON damage, the average peak IOPs were 35.70 ± 4.45 mm Hg in treated eyes compared with 16.78 ± 1.21 mm Hg in the companion eyes (values are the mean ± SD; $n = 14$; Fig. 1). In animals with induced ocular hypertension plus intraocular injection of MIO-M1 cells, the average peak IOPs were 43.71 ± 9.16 mm Hg in treated eyes compared with 16.46 ± 3.01 mm Hg in the contralateral eyes (values are mean ± SD; $n = 48$; Supplementary Fig. S1). After 4 weeks, this glaucoma model produced 34.5% ± 31.7% RGC axonal loss, as assessed by semiquantitative analysis of optic nerve axonal survival, which is similar to that achieved by others.[33]

Survival and Migration of Transplanted MIO-M1 Cells

Transplanted MIO-M1 cells were tracked in vivo by their expression of EGFP and human nuclear antigen. MIO-M1 cells transplanted into the vitreous of glaucomatous survived well after 1 week (10/12 grafts; Figs. 2A, 2B), 2 weeks (17/30 grafts; Figs. 2C, 2D), and 3 weeks (2/3 grafts; data not shown) in vivo. Generally, engrafted cells transplanted into the posterior vitreous adjacent to the retina remained as a discrete bolus and did not disperse extensively. MIO-M1 cells exhibited an elongated, bipolar, migratory-like morphology at all times points and were frequently oriented perpendicular to the retina. Furthermore, intravitreally engrafted MIO-M1 cells survived in nonglaucomatous eyes 2 weeks after injection and displayed a morphology similar to those within the injured eye (2/4 grafts; Figs. 2E, 2F and Supplementary Fig. S2). Although the immunosuppressive regimen used greatly improved the survival of transplanted cells, rejection was delayed rather than prevented. Grafts were attacked by microglia/macrophages at all time points, as revealed by immunohistochemical staining against the markers CD68 and CD11b (Figs. 2G, 2H, 2I). MIO-M1 cells were consistently destroyed by 4 weeks in vivo. A time point of 2 weeks in vivo was subsequently used, as it allowed a compromise between maximum graft health and time in vivo.

Not all MIO-M1 cells retained their EGFP expression. Immunohistochemical labeling revealed many human nuclear antigen–positive cells within discrete grafts that were GFP-negative (Figs. 2A, 2B, 2C, 2D). Retrospective analysis of MIO-M1 cells in vitro confirmed that only about half of the transplanted cells were EGFP-positive (data not shown), although expression in the remaining cells was bright. Both EGFP and human nuclear antigen reactivity were used to track transplanted cells, although the signal from human nuclear antigen labeling was faint, and use of the additional marker restricted immunohistochemical double labeling.

MIO-M1 cells were also transplanted subretinally in the glaucomatous eye (Fig. 2J). This method of delivery proved to be much less reliable, at least in our hands, than intravitreal targeted injections. Although the injection site could usually be identified, as it caused a retinal detachment, few MIO-M1 cells survived at either 1 (2/14 grafts) or 2 (8/28 grafts) weeks after injection. Extensive invasion by immune cells at this site was also observed.

In Vivo Integration and Differentiation of MIO-M1 Cells

MIO-M1 cells were not found to migrate into the glaucomatous or uninjured adult retina after either intravitreal or subretinal delivery (Figs. 2A–J), within the time frame delineated by immune rejection. Even when a graft traversed all layers of the retina, no EGFP-positive or human nuclear antigen–positive cells were observed to leave the graft and migrate into any of the retinal layers, despite direct contact (Figs. 2C, 2D). Furthermore, exposure of cells to extracellular matrix protein and basic fibroblast growth factor (bFGF) in vitro, before intraocular injection, did not improve their migratory capacity or ability to integrate into the retina (data not shown).

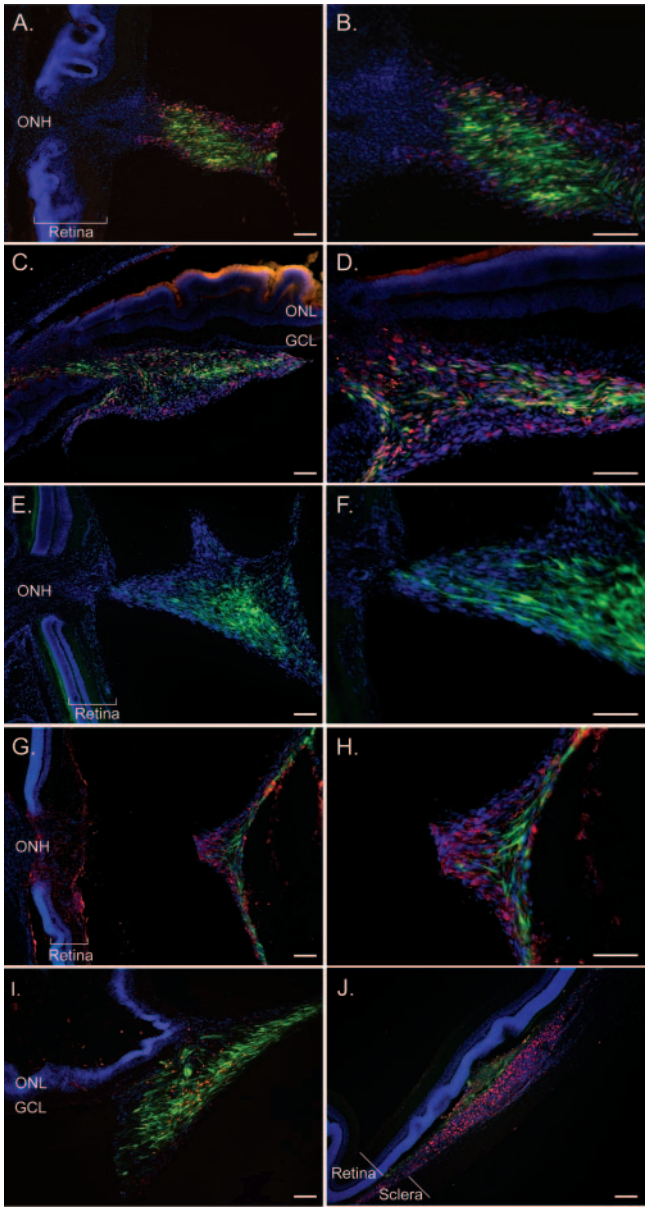

FIGURE 2. Survival and migration of MIO-M1 cells after transplantation into the eye. (**A**, **B**) MIO-M1 cells transplanted intravitreally (GFP; *green*) survived well at 1 week after transplantation into the glaucomatous eye. Immunohistochemical labeling for human nuclear antigen (*red*) revealed that many cells of human origin were not EGFP-positive. Sections were counterstained with DAPI (*blue*). Image (**A**) is magnified in (**B**). (**C**, **D**) Engrafted MIO-M1 cells (EGFP; *green*) survived for 2 weeks after injection into the vitreous of the glaucomatous eye. Again, labeling for human nuclear antigen (*red*) revealed that many transplanted cells were not GFP-positive. Sections were counterstained with DAPI (*blue*). Furthermore, although this transplant was intended to be intravitreal, by chance it also crossed all retinal layers through the injection site and clearly demonstrated the failure of MIO-M1 cells to migrate into the retina. Image (**C**) is magnified in (**D**). (**E**, **F**) Intravitreal graft of MIO-M1 cells (EGFP; *green*) 2 weeks after transplantation into a nonglaucomatous control eye and stained with DAPI (*blue*). Transplanted cells displayed a similar morphology and migratory potential in both control and injured eyes. Image (**E**) is magnified in (**F**). (**G**, **H**) Immunologic rejection of intravitreally engrafted MIO-M1 cells (EGFP; *green*) was also observed at all time points (sections from 2 weeks after transplantation shown) by labeling for the macrophage/microglial marker CD11b (*red*). Extensive microglial activation and macrophage invasion were common in transplant-recipient eyes. Sections were counterstained with DAPI (*blue*). Image (**G**) is magnified in (**H**). (**I**) Labeling of intravitreally engrafted MIO-M1 cells (EGFP; *green*), 2 weeks after transplantation into a glaucomatous eye, with an antibody against the macrophage/microglia marker CD68 (*red*) confirmed CD11b immunohistochemistry results. Sections counterstained with DAPI (*blue*). (**J**) Subretinal graft of MIO-M1 cells (EGFP; *green*) 2 weeks after transplantation into a glaucomatous eye labeled for CD68 (*red*) and counterstained with DAPI (*blue*). Very few MIO-M1 cells remained in the graft, and extensive macrophage invasion was observed. ONH, optic nerve head; ONL, outer nuclear layer; GCL, ganglion cell layer. Scale bar, 100 μm.

After 2 weeks in vivo, many intravitreal grafts contained MIO-M1 cells immunopositive for the neuronal marker β-III-tubulin (Figs. 3A–C) that exhibited fine, bipolar processes. The expression of β-III-tubulin was found to vary greatly between grafts: some had many β-III-tubulin–positive cells (Fig. 3C), whereas others had comparatively few (Fig. 3B). Very rarely, single EGFP-positive MIO-M1 cells were observed with a complex, fully differentiated, neuronal-like morphology (Fig. 3D). Similar behavior by MIO-M1 cells was observed in uninjured eyes (Supplementary Fig. S2).

MIO-M1 cells also demonstrated a capacity to express glial cell markers in vivo. Two weeks after transplantation, a minority of engrafted cells expressed the glial cell marker GFAP (Figs. 3E, 3F, and Supplementary Fig. S2). Expression of this protein was also upregulated in both astrocytes and Müller cells within the injured retina, as observed previously in glaucoma.[40] GFAP-positive cells displayed a radial morphology not dissimilar to that of the β-III-tubulin-positive cells also observed within grafts. However, despite a similarity in morphology, the expression of β-III-tubulin and GFAP by MIO-M1 cells in vivo was not found to colocalize (Fig. 3G).

The elongated, bipolar morphology displayed by MIO-M1 cells after transplantation into the eye was reminiscent of a migratory neuroblast phenotype. Immunohistochemical analysis of engrafted MIO-M1 cells revealed limited expression of PSA-NCAM, a marker common to migrating neuroblasts (Fig. 3H).

Extracellular Matrix Modulation to Enhance MIO-M1 Integration

To encourage the migration of MIO-M1 cells into the tissue, we coadministered either chondroitinase ABC or erythropoietin (EPO) in an attempt to modify the inhibitory retinal environment. Application of these factors was observed to help approximately 18 to 30 $EGFP^+$-MIO-M1 cells/eye (calculated as three to five cells/1:6 series of posterior eyecup sections) enter the glaucomatous retina, whereas previously such cells were never observed within the retina (Figs. 4A–G). It should be noted that this count is likely to be greatly underestimated, given that less than half of the MIO-M1 cells injected into the eye expressed the marker protein EGFP (as discussed above; Fig. 2). Exposure of the retina to EPO permitted cells to enter the retina from either the vitreous (Figs. 4B–D; observed in one of three surviving grafts) or subretinal space (Fig. 4A; one of one surviving grafts). EGFP-positive cells were seen to extend into the retina, particularly from the vitreous side. In the presence of EPO, some MIO-M1-derived cells extended processes from the ganglion cell layer through the inner retina to the outer nuclear layer (Fig. 4B). Colabeling for the microglia/macrophage marker CD68 confirmed that the integrated cells were not macrophages/microglia that had engulfed dead GFP-positive cells or debris (Figs. 4A, 4B). Similarly, coinjection of chondroitinase ABC with MIO-M1 cells into the glaucomatous eye greatly enhanced the ability of the transplanted cells to invade the retina (Figs. 4E, 4G). When injected subretinally, EGFP-positive cells migrated into the retina and extended long,

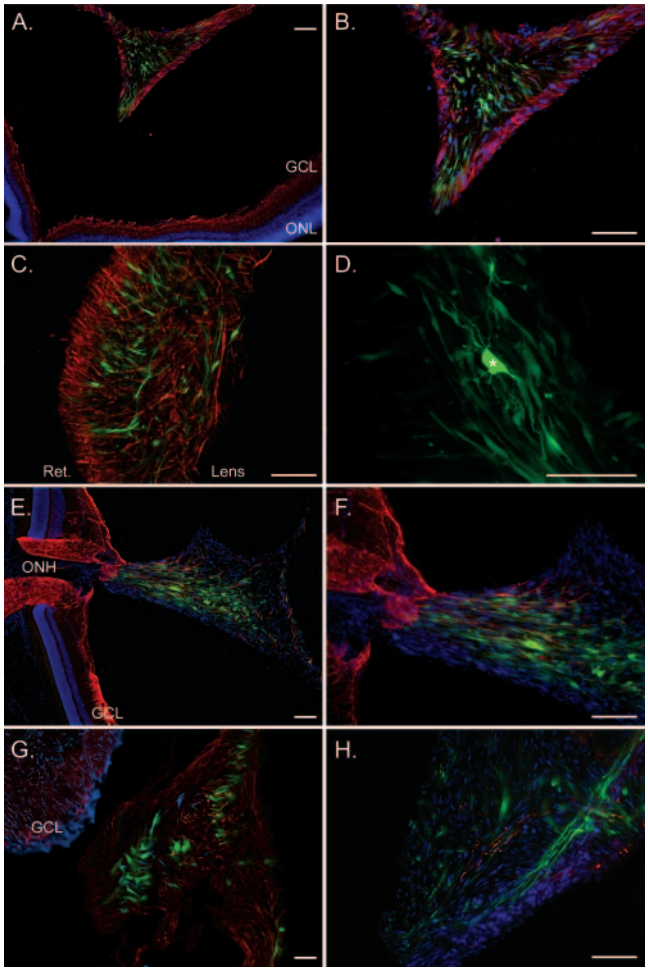

FIGURE 3. Integration and differentiation of MIO-M1 cells 2 weeks after transplantation into the glaucomatous rat eye. (**A–C**) Engrafted MIO-M1 cells (EGFP; *green*) immunohistochemically labeled for β-III-tubulin (*red*) and stained with DAPI (*blue*). Grafts showed variability in expression of the neuronal marker β-III-tubulin, with some grafts producing many (**C**) and others fewer (**B**) β-III-tubulin-positive cells. In addition, transplanted cells commonly displayed an elongated morphology reminiscent of migrating neuroblasts. β-III-Tubulin expression by neurons within the inner retina was also observed, as expected. The graft depicted in (**C**) is intravitreal and distant from the retina (Ret. retinal side of the graft; Lens, lens side). Image (**A**) is magnified in (**B**). (**D**) Rarely, transplanted MIO-M1 cells (EGFP; *green*) displayed a neuronal-like phenotype (✶) within the graft and possessed fine neurite-like extensions. (**E**, **F**) Some of the transplanted MIO-M1 cells (EGFP; *green*) were observed to express the astrocytic/Müller cell marker GFAP (*red*). In the injured retina, GFAP was also expressed by astrocytes and Müller cells, as expected. Counterstain was DAPI (*blue*) and image (**G**) is magnified in (**H**). (**G**) Within grafts, there was no evidence that β-III-tubulin (*red*) and GFAP (*blue*) were coexpressed by MIO-M1 cells (EGFP; *green*). (**H**) Some cells within intravitreal MIO-M1 grafts (EGFP; *green*) were PSA-NCAM (*red*) immunoreactive; however, they were few, although many cells within the grafts displayed an elongated, migratory morphology. Counterstain was DAPI (*blue*). ONH, optic nerve head; ONL, outer nuclear layer; GCL, ganglion cell layer. Scale bar, 100 μm.

neuritelike processes deep into the outer nuclear layer (Figs. 4E, 4F; three of three surviving grafts). In addition, chondroitinase ABC aided the migration of intravitreally transplanted cells into the ganglion cell layer, from where they also extruded long extensions into the inner retina (Fig. 4G; one of three surviving grafts). Immunohistochemical labeling for CD68 and CD11b again confirmed that the integrated EGFP-positive cells were not macrophages/microglia (Figs. 4E, 4G). Chondroitinase ABC activity within the retina was confirmed using the 1B5-clone antibody, which recognizes the remaining proteoglycan "stub" after chondroitinase ABC-mediated proteoglycan

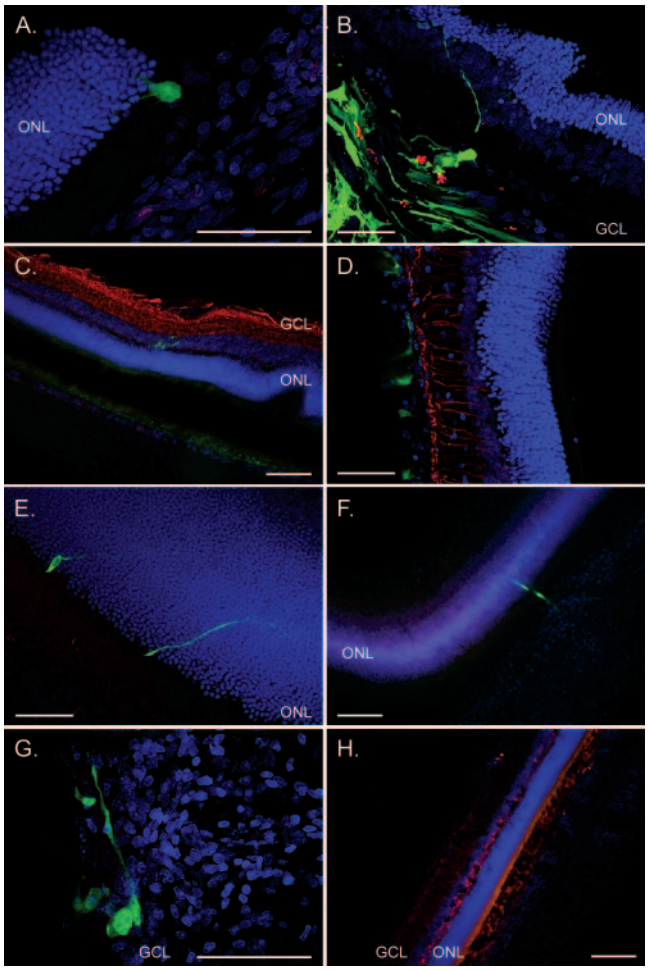

FIGURE 4. Treatment with either EPO or chondroitinase ABC facilitated retinal integration of MIO-M1 cells in the glaucomatous eye after 2 weeks in vivo. (**A–D**) Intraocular injection of EPO simultaneously with MIO-M1 cells (EGFP; *green*) enhanced the ability of the grafted cells to migrate into the retina. This benefit was observed whether the cells were transplanted subretinally (**A**) or intravitreally (**B**, **C**). Immunohistochemical labeling for CD68 (*red*) demonstrated that the engrafted cells were not simply microglia/macrophages that had engulfed GFP^+ cell debris (**A**, **B**). Despite migration into the retina, the MIO-M1 cells did not express the neuronal marker β-III-tubulin, although it was expressed by inner retinal neurons as expected (**C**; *red*). Müller cells within the engrafted retina were immunoreactive for nestin (**D**); the antibody used to detect nestin was nonreactive for human nestin. Sections were counterstained with DAPI (*blue*). (**E–G**) Intraocular administration of chondroitinase ABC also aided MIO-M1 (EGFP; *green*) migration into the glaucomatous retina. When transplanted subretinally, engrafted cells were observed sending neurite-like extensions into the outer retina (**E**, **F**). Likewise, intravitreal transplantation allowed MIO-M1 processes to invade the inner retina (**G**). Immunohistochemical probing for CD68 or CD11b reactivity (**E**, **G**, respectively) revealed that the engrafted cells were not $EGFP^+$ macrophages. Despite migration into the retina, these cells did not express the neuronal marker β-III-tubulin (**F**, *red*). Tissue was counterstained with DAPI (*blue*). (**H**) Chondroitinase ABC activity was confirmed using the 1B5 antibody directed against proteoglycan cleavage products (*red*). Patchy staining was observed throughout the retina, particularly within the plexiform layers. Tissue was counterstained with DAPI (*blue*). ONH, optic nerve head; ONL, outer nuclear layer; GCL, ganglion cell layer. Scale bar, 100 μm.

cleavage (Fig. 4H). Cleavage product was observed mainly within the retinal plexiform layers, in particular the outer nuclear layer. Given that the tissue was taken 2 weeks after enzyme injection, it is possible that enzyme activity was greater immediately after application.

The differentiation of MIO-M1 cells after their integration into the glaucomatous retina was also examined. Surprisingly, despite displaying an elongated, neuronal-like morphology, none of the integrated cells expressed markers suggestive of a mature phenotype. In particular, the neuronal marker β-III-tubulin was not expressed by any integrated GFP-positive cells, whether they were delivered subretinally or intravitreously (Fig. 4C, 4F, respectively). In addition, none of the integrated cells were observed to express the classic glial marker GFAP (data not shown).

Discussion

Stem cell–based therapeutic approaches are of potential interest in progressive degenerative diseases such as glaucoma, in which loss of a single population of accessible neurons results in disability. Although stem cell transplantation has been shown to reduce neuronal loss and replace outer retinal neurons in some retinal disease models,[12,23–26,41] stem cell–based therapies have not been explored for the treatment of glaucoma. In the present study, we investigated the potential of MIO-M1 cells, a human Müller cell line that possesses stem cell–like qualities, for the future development of a novel therapy for glaucoma. These cells have been well characterized[6] and were recently tested in other neurodegenerative ocular diseases.[32] We found that MIO-M1 cells can survive well in vivo within the eye and respond to their environment, as demonstrated by their tendency to orient toward the retina and, particularly, the optic nerve head. Unfortunately, the in vivo timeframe of our experiments was limited to 2 to 3 weeks because of rejection of the xenograft, despite constant administration of a cocktail of immune suppressant drugs. This prevented long-term tracing of engrafted cells to explore their in vivo potential fully, as it typically takes much longer for precursor cell differentiation and integration within the adult CNS.[10,28,30,31] Furthermore, at least half of cells from the MIO-M1 cell line lost their engineered EGFP expression, which hindered tracking of all the transplanted cells. Reliable isolation of cells equivalent to MIO-M1 from rats, which has proven difficult to date, would facilitate the investigation of stem cell therapy applications for glaucoma, as this may eliminate the immune and innate phagocytic responses caused by xenograft rejection, which may have played an important role in this study.

Unlike the brain, it appears that the adult retina does not provide a permissive environment in which transplanted stem cells can easily migrate and integrate. Previous studies have found almost no integration of stem cells into the adult uninjured retina[10,20] but improved permissibility within the injured retina.[21,22] Of interest, MIO-M1 cells responded similarly in both injured and uninjured eyes. MIO-M1 cells were found to exhibit bipolar, migratory-like phenotypes that often aligned toward the retina, in particular the optic nerve head, in both uninjured and glaucomatous eyes. However, despite the morphologic suggestion of migratory potential, MIO-M1 cells typically did not disperse extensively within the eye and tended to remain as a discrete graft. This result suggests that the cells either lack the ability to migrate, for example perhaps they do not express appropriate proteins, or they may form a self-contained niche from which it is difficult for them to exit. It is possible that such limitations may be overcome by manipulating the cells in vitro before transplantation. Furthermore, such interactions could be reduced by transplanting fewer cells, although the number of cells delivered in this study was comparable to or less than previous investigations where dispersal was more successful, albeit using different cell types and different disease models.[10,12,21,28]

We have found that the retina, both normal and glaucomatous but without extracellular matrix modification, did not permit the integration of MIO-M1 cells when delivered either intravitreally or subretinally. Furthermore, we demonstrated that even when in direct contact with all retinal layers MIO-M1 cells were unable to penetrate the mature retina. Given that other types of stem cells, most notably embryonic stem cells,[7,30,31] can migrate into the injured retina, the lack of integration by MIO-M1 cells may suggest a deficit peculiar to this cell line. We know from previous in vitro characterization that these cells exhibit stem cell–like properties and are multipotent.[6,32] Recently, it was demonstrated that MIO-M1 cells, and two other similar cell lines, could integrate into the retina of neonatal and injured adult RCS rats, where they differentiated into various cell types.[32,37] We used equivalent culture conditions and exposure to extracellular matrix plus bFGF, previously found to enhance migration, but we did not see evidence of retinal integration in our model without additional modulation of the retinal environment. Furthermore, extracellular matrix digestion was observed to aid integration but was not necessary.[32,37] Indeed, even the cells that survived in vivo displayed a strikingly different morphology, being bipolar with very elongated extensions rather than small and spheroid-shaped, as observed beforehand. Previously, it was noted that suppression of immune reactivity enhanced MIO-M1 retinal integration.[37] Therefore, it is plausible that use of a different rat strain and model affected the degree of tissue reactivity to which the engrafted cells were exposed. This, in turn, may have inhibited their ability to migrate into the glaucomatous retina. Significant strain-dependent differences in tissue plasticity have recently been highlighted in an elegant study that found that inhibition of T-cell activity in two rats strains, known to differ in their susceptibility to autoimmune disease, modulated the survival and regeneration of axotomized RGCs.[42]

In an effort to overcome the natural repulsion of MIO-M1 cells by the retina, we attempted to modify the local environment by application of either EPO or chondroitinase ABC. EPO has been shown to upregulate expression of matrix metalloproteinase 2 (MMP2) within the CNS,[43] which in turn may facilitate retinal integration.[44] Furthermore, EPO has been reported to enhance the migratory potential of proliferating Müller cells in the retina.[45] Chondroitinase ABC has been investigated as a means of modifying the mature central nervous system environment to encourage plasticity.[46] The enzyme degrades cell surface chondroitin sulfate proteoglycans that have been shown to play a role in stabilizing the mature neural environment to prevent axonal/dendritic sprouting.[47] In addition, pretreatment with chondroitinase ABC has been reported to aid the integration of transplanted cells into the mature CNS.[48] A promising finding in our study was that a single application of either EPO or chondroitinase ABC facilitated the migration of MIO-M1 cells into the glaucomatous retina where no integration was previously observed. Simultaneous injection of either EPO or chondroitinase ABC with MIO-M1 cells resulted in a small number of GFP-positive MIO-M1 cells entering the injured retina and extending fine processes across multiple retinal layers. In particular, chondroitinase ABC appeared to promote long neurite extension, an effect noted previously when used to enhance axonal regeneration in the injured spinal cord.[49,50] Although few integrated MIO-M1 cells were observed, it is possible that they were underestimated, as at least half of the cells had lost expression

of the EGFP marker, over time in vitro, before implantation. However, the successful movement of transplanted cells into the retina demonstrated that it is possible to modify the in vivo environment to enhance the integration of transplanted cells, which may facilitate the development of future therapies.

We also examined the ability of MIO-M1 cells to differentiate in the glaucomatous eye when cells were delivered either intravitreally or subretinally. We found an intravitreous route to be much more reliable than subretinal delivery, which frequently resulted in graft failure and MIO-M1 death. It is unclear why the subretinal injections were problematic, as this approach has previously been used successfully.[12,32] However, it must be noted that intravitreal injection was not without problems, as it was often difficult to place the cells precisely adjacent to the retina. Perhaps alternative delivery methods, for example a scaffold delivery system instead of an injection, would be more useful for this cell type. Indeed, alternative delivery mechanisms have been found very efficacious for the transplantation of cells in other systems, as recently reviewed.[51] Despite these limitations, we have demonstrated that MIO-M1 cells are capable of differentiating toward a neuronal phenotype, as many engrafted cells expressed the marker β-III-tubulin after 2 weeks in vivo. Indeed, MIO-M1 cells appeared more inclined to neuronal rather than glial differentiation, as few cells expressed the glial marker GFAP after engraftment. This finding is supported by recent results demonstrating that similar cell lines can differentiate into a variety of neuronal cell types after injection into the neonatal rat's eye.[32] Engrafted $EGFP^+$ cells only rarely displayed a mature neuronal morphology; however, this does illustrate their potential to generate neurons in vivo, an ability that may have been more successful given more time. In addition, despite exhibiting a morphology reminiscent of migrating neuroblasts, transplanted MIO-M1 rarely expressed PSA-NCAM, a marker associated with migrating cells in the CNS. Although strong expression of β-III-tubulin and some expression of GFAP by MIO-M1 cells within discrete intravitreal grafts was common, such expression by EGFP-positive cells integrated into the retina was not observed. However, perhaps such cells may have continued to differentiate if provided with more time in vivo, which was unfortunately limited by xenograft rejection. Alternatively, it may not be necessary for MIO-M1 cells to differentiate into fully mature neurons to provide a beneficial therapy in glaucoma. Many studies have now demonstrated an inherent ability of various stem cell types to support neurons and ameliorate neurodegenerative processes within the CNS.[14,16,17,19] It is not clear exactly how this effect is mediated, although research suggests it may be through the provision of trophic factors to stressed neurons. In support of this hypothesis, engineered overexpression of various factors has been found to enhance stem cell–mediated neuroprotection.[11,15,52–56] Such neuroprotection remains to be tested in glaucoma.

In summary, we have demonstrated that the human-derived Müller cell progenitor cell line, MIO-M1, is capable of surviving within the glaucomatous eye and of acquiring neural morphology upon intravitreal transplantation. Furthermore, we have found that concomitant delivery of either EPO or chondroitinase ABC with engrafted cells significantly enhances their ability to migrate into the adult retina. In particular, modification of the retinal environment with chondroitinase ABC facilitated the entry of MIO-M1 cells into the mature retina and their extension of fine neurites across multiple retinal layers. Together, these results suggest that stem cells derived from the neural retina may be useful in the development of novel therapies for the treatment of glaucoma. The effects of these cells on existing neuronal circuits and their ability to provide neuroprotection for remaining RGCs to slow disease progression are important topics for future investigation.

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