Mesenchymal Stem Cell–Derived Small Extracellular Vesicles Promote Neuroprotection in Rodent Models of Glaucoma

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Submitted: August 23, 2017
Accepted: January 4, 2018
Citation: Mead B, Amaral J, Tomarev S. Mesenchymal stem cell–derived small extracellular vesicles promote neuroprotection in rodent models of glaucoma. Invest Ophthalmol Vis Sci. 2018;59:702–714. https://doi.org/10.1167/iovs.17-22855

PURPOSE. To investigate the benefit of bone marrow mesenchymal stem cell (BMSC)-derived small extracellular vesicles (sEV) as an intravitreal (ivit) therapy in two rat models of glaucoma and to determine and identify candidate miRNA involved in the mechanism.

METHODS. sEV were isolated from human BMSC and fibroblasts and ivit injected into adult rats after induction of elevated IOP. IOP was elevated using either intracameral injection of microbeads or laser photocoagulation of circumferential limbal vessels and the trabecular meshwork. Retinal nerve fiber layer (RNFL) thickness was measured using optical coherence tomography, positive scotopic threshold response (pSTR) recorded using ERG, and RNA binding protein with multiple splicing (RBPM3+) retinal ganglion cell (RGC) counted using retinal wholemounts. sEV miRNA were sequenced using RNAseq.

RESULTS. sEV isolated from BMSC promoted significant neuroprotection of RGC while preventing RNFL degenerative thinning and loss of pSTR. sEV proved therapeutically efficacious when ivit injected every week or every month, but ineffective with longer delays between treatments. Knockdown of Argonaute2 (AGO2), a protein critical for miRNA function and packing into sEV prior to sEV isolation, significantly attenuated the above effects. Addition of BMSC sEV (but not fibroblast sEV) reduced death of cultured purified RGC. RNAseq identified 43 miRNA upregulated in BMSC sEV in comparison to fibroblast sEV, which yielded no neuroprotective effects.

CONCLUSIONS. Injection of BMSC-derived sEV into the vitreous provided significant therapeutic benefit to glaucomatous eyes. The neuroprotective effect of sEV, at least partially, may be explained by direct action on RGC through miRNA-dependent mechanisms.

Keywords: glaucoma, mesenchymal stem cells, extracellular vesicles, exosomes, retinal ganglion cells, miRNA

Glaucoma is one of the world’s leading causes of irreversible blindness and is characterized by the slow progressive degeneration of retinal ganglion cells (RGC) and their axons.1 RGC operate as the final stage in the phototransductive visual pathway of the retina, tasked with the projection of electrochemical information to the brain along their axons, which make up the optic nerve. RGC are irreplaceable, making their dysfunction and subsequent loss a severe detriment to vision and thus, quality of life. While current therapies can successfully reduce IOP, the critical risk factor associated with glaucoma, no neuroprotective strategies currently exist.

Mesenchymal stem cells (MSC) are a multipotent stromal cell residing in mesenchymal tissues such as bone marrow (BMSC),2 adipose (ADSC),3 umbilical blood (UCB-MSC),4 and dental pulp.5 Their neuroprotective efficacy in retinal injury models has been demonstrated by us6–9 as well as independent research groups,10–12 and are currently undergoing clinical trials for retinal degenerative diseases such as glaucoma.13 The mechanism of action is exclusively paracrine, through the secretion of neuroprotective factors as opposed to RGC replacement. MSC secrete a large abundance of factors, of which several neuroprotective candidates have been identified including brain-derived neurotrophic factor (BDNF) and platelet-derived growth factor (PDGF).7,14

Extracellular vesicles (EV) include exosomes, microvesicles, and apoptotic bodies and were described decades ago as being secreted from most cell types both by outward budding of the plasma membrane as well as intracellular formation within multivesicular endosomes before secretion into the extracellular space.15 The formation of the vesicle as well as the loading of cargo is reliant on a complex of 30 proteins referred to as Endosomal Sorting Complex Required for Transport (ESCRT). Exosomes contain mRNA, miRNA, lipids, and protein and can be easily isolated from various bodily fluids as well as conditioned medium in vitro.15,16 Exosomes are typically defined by their size of less than 150 nm although a 220 nm threshold is often used as the sample is passed through 220-nm porous filter.17 As this preparation includes nonexosomal EV, this population will be referred to as small EVs (sEV) from this point. Originally thought to be a mechanism solely for the
Extracellular Vesicle Treatment for Glaucoma

Adapted for the Use of Animals in Ophthalmic and Vision Research, using protocols approved by the National Eye Institute Committee on the Use and Care of Animals.

Animals were kept at 21°C and 55% humidity under a 12-hour light and dark cycle, given food/water ad libitum and were under constant supervision from trained staff. Animals were euthanized by rising concentrations of CO₂ before extraction of retinae.

Materials

All reagents were purchased from Sigma (Allentown, PA, USA) unless otherwise specified.

BMSC Cultures

Human CD29<sup>+</sup>/CD44<sup>+</sup>/CD73<sup>+</sup>/CD90<sup>+</sup>/CD45<sup>+</sup> BMSC (confirmed by supplier; Lonza, Walkersville, MD, USA) from three donors were pooled and cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 1% penicillin/streptomycin and 10% exosome-depleted fetal bovine serum (Thermo Fisher Scientific, Cincinnati, OH, USA). Cell cultures were maintained at 37°C in 5% CO₂ with medium changed every 3 days and cells passaged with 0.05% trypsin/EDTA when 80% confluent. Human dermal fibroblasts (Lonza) were grown in the above conditions and used as a control. For all experiments, BMSC and dermal fibroblasts were used at passage two through five.

Transfection and Confirmation of Knockdown

For a subgroup of animals, BMSC were transfected using Lipofectamine 3000 (Thermo Fisher) per the manufacturer’s protocol. Briefly, 70% confluent BMSC grown in Opti-MEM medium were incubated with Lipofectamine 3000 reagent and either siRNA against AGO2 (SiAgo2, #4392420/assay id s25931; Thermo Fisher Scientific) or a scrambled control siRNA (#4390843; SiScr) for 48 hours. AGO2 knockdown (>70%) was confirmed by Western blotting like previously described.<sup>22</sup> (Supplementary Fig. S1).

Exosome/sEV Isolation and Quantification

Exosomes were isolated from BMSC and fibroblasts using ExoQuick-TC (System Biosciences, Mountain View, CA, USA) per the manufacturer’s instructions. Briefly, conditioned medium was centrifuged at 3000g for 15 minutes to remove cells and debris, incubated with ExoQuick reagent overnight at 4°C (1:10 ratio with medium), centrifuged at 1500g for 15 minutes a final time before the exosome pellet is resuspended in sterile PBS. The exosome preparation is passed through a 0.22-μm filter to remove any large extracellular vesicles (microvesicles and apoptotic bodies). Because it is expected some nonexosomal vesicles remain in the preparation, we refer to the exosomes used in this study as sEV. Using Western blot, exosomes were characterized by their positive staining for the exosome/sEV markers Syntenin-1 and CD63 and negative staining for high-/low-density lipoprotein markers ApoA1 and ApoB (Supplementary Fig. S2). Briefly, sEV were lysed in passive lysis buffer (#E1531; Promega, Madison, WI, USA) before protein concentration was determined by BCA protein assay (Thermo Fisher). Protein samples (20 μg) were separated on 4% to 12% Bis-Tris protein gels at 150 V for 40 minutes. Proteins were transferred to polyvinylidene fluoride membranes, blocked in 10% Western blot blocking buffer (Roche, Basel, Switzerland) in Tris-buffered saline (TBS), stained overnight in primary antibody (Table 1) diluted in TBS, washed 3 × 5 minutes in TBST, stained for 1 hour with secondary

METHODS

Animals

Adult female outbred Sprague-Dawley rats weighing 150 to 200 g (Charles River, Wilmington, MA, USA) were maintained in accordance with guidelines described in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, using protocols approved by the National Eye Institute Committee on the Use and Care of Animals.

Animals were kept at 21°C and 55% humidity under a 12-hours light and dark cycle, given food/water ad libitum and were under constant supervision from trained staff. Animals were euthanized by rising concentrations of CO₂ before extraction of retinae.

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antibody (Table 1) in TBS, washed 5 × 5 minutes in TBST before detection with Immobilon ECL reagents (Millipore, Burlington, MA, USA). Densitometry of Western blot bands were analysed using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). sEV were derived from BMSC pooled from 3 donors and this pooled sample of sEV was assayed in triplicate by Western blot and used throughout the remainder of the study.

The concentration and size distribution of sEV were characterized using a NanoSight LM10 instrument (Malvern, Worcester, MA, USA), equipped with a 405 nm LM12 module and EM-CCD camera (DL-658-OEM-630; Andor, Concord, MA, USA). Three videos were captured per sample with a camera level of 10. Videos were analyzed with a detection threshold of two, automatic blur size and 12.9- to 13.1-pix maximum jump size. Slider gain was set to 80 and a total of 567 frames were taken.

### Table 1. Antibodies Used in Immunohistochemistry (IHC), Immunocytochemistry (ICC), and Western Blot (WB)

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<th>Antigen</th>
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In Vivo Experimental Design

The experimental design is shown schematically in Figure 1A. Seventy rats were divided into three groups: Group 1 consisted of five uninjured/untreated animals; Group 2 consisted of 30 rats with ocular hypertension induced by intracameral (ic) injection of microbeads; Group 3 consisted of 35 rats with ocular hypertension induced by laser photocoagulation of the trabecular meshwork (TM) and limbal vessels. Induction of the model as well as treatment with ivit sEV (BMSC or fibroblasts) were performed bilaterally and began on day 0 with some animals further receiving a weekly treatment. While it is not possible to treat Group 3 monthly, as the experimental length is 21 days, Group 2, which is a 56-day experiment also received monthly injections. Along with BMSC and fibroblast sEV treatments, Group 3 also received sEV derived from MSC transfected with SiAgo2 or SiScr. To make up for any animals/tissue that were unusable at the end of the study (due to animal deaths, surgical problems, lack of ocular hypertension, or wholemounting errors), additional animals were run to ensure each treatment subgroup was made up of five animals/10 eyes.

### Induction of Ocular Hypertension With Intracameral Microbeads

Ocular hypertension was induced in Group 2 by ic injection of microbeads as previously described. Anesthesia was induced with 5% Isoflurane (Baxter Healthcare Corp, Deerfield, IL, USA)/1.5 L per minute O2 and maintained at 3.5% throughout the procedure. Using a 15° blade (Fine Science Tools, Reading, PA, USA) a small 2-mm incision was made at the peripheral cornea and aqueous humour was allowed to exude. Using the same incision site, a 10-μL solution of microbeads was administered with a glass micropipette, produced in-house from a glass capillary rod (Harvard Apparatus, Kent, UK) using a Flaming-Brown micropipette puller (Sutter Instruments, Novato, CA, USA). The microbead solution was loaded into the microneedle immediately before injection and consisted of 5 μL of 6-μm beads (polystyrene, Cat.#7512; Polysciences, Inc., Warrington, PA, USA) followed by 5 μL of 10-μm beads (polystyrene, Cat.#7156; Polysciences, Inc.), both at concentrations of 2 × 10^5/mL. Administration was made slowly and the needle was retracted with a 2-minute delay to minimize leakage. Due to the variable translucency of
Figure 1. Experimental design of study and exosome isolation. (A) Timeline and groupings of study, detailing the number of animals (eyes) per group, the two glaucoma models used and the sEV treatment schedule. (B) Nanosight analysis of extracellular vesicles isolated from BMSC, demonstrating their size and relative quantity.
The eye after microbead injection, reliable ERG and optical coherence tomography (OCT) measurements were not possible.

**Induction of Ocular Hypertension With Laser Photocoagulation**

Ocular hypertension was induced in Group 3 by laser photocoagulation of the trabecular meshwork/limbal vessels and ivit sEV treatments. Asterisks represent significant difference between intact/control and experimental groups (P < 0.05).

**Intravitreal Delivery of sEV**

Under isoflurane-induced anesthesia, sEV were injected into the vitreous, just posterior to the limbus using glass micropipette. A 5-µL volume of sterile phosphate-buffered saline (sPBS) loaded with 3 × 10⁹ sEV was injected slowly and the needle was retracted after a 1-minute delay to minimize backflow. The concentration was chosen based on our previous study that demonstrated efficacy.

**Electroretinography Measurements of the Positive Scotopic Threshold Response**

ERG was recorded using the Espion Ganzfeld full-field system (Diagnosys LLC, Lowell, MA, USA) on day 0 before induction of ocular hypertension, and on day 56/21 (Groups 2 and 3, respectively) before animals were killed. Rats were dark adapted for 12 hours overnight and prepared for ERG recording under dim red light (>650 nm). Anesthesia was induced with intraperitoneal injection of ketamine/xylazine and eyes dilated with tropicamide. Scotopic flash ERG was recorded from −5.5 to +10 log units with respect to standard flash in half-log-unit steps. ERG traces were analyzed using built Espion software and the amplitude (with respect to baseline) was used as a measure of rat visual function. Traces at a light intensity of 1 × 10⁻⁵ mcd/s were chosen for analysis as they gave a clean, unambiguous pSTR 100 ms after stimulus. An individual masked to the treatment groups performed all readings and analysis.

**Optical Coherence Tomography Measurements of the Retinal Nerve Fiber Layer**

OCT was performed on rats under anesthesia (intraperitoneal ketamine/xylazine) on day 0 before induction of ocular hypertension, and on day 56/21 (Groups 2 and 3, respectively) before animals were killed. A Spectralis HRA3 confocal scanning laser ophthalmoscope (Heidelberg Engineering, Heidelberg, Germany) was used to take images of the retina around the optic nerve head and in-built software segmented the retinal nerve fiber layer (RNFL) and quantified the thickness. Segmentation was manually adjusted (by an individual masked to the treatments groups) when necessary to prevent inclusion of blood vessels that populate the RNFL.

**RGC Counts in Retinal Wholemounts**

Rats were euthanized at 56/21 days (Groups 2 and 3, respectively) by rising concentration of CO₂ and perfused intracardially with 4% paraformaldehyde (PFA) in PBS. Eyes were enucleated and retinæ dissected and immersion post-fixed in 4% PFA for 1 hour at 4°C. Wholomounted retinæ were permeabilized in 0.5% Triton X-100 in PBS for 15 minutes at −76°C, washed in fresh Triton X-100 for a further 15 minutes before incubation with primary antibody diluted in whole-mount antibody diluting buffer (wADB2% bovine serum albumin, 2% Triton X-100 in PBS) overnight at 4°C and, the following day, were washed 3 × 10 minutes in PBS and incubated with secondary antibodies in wADB for 2 hours at room temperature. After 2 hours, retinæ were washed for 3 × 10 minutes in PBS and mounted vitreous side up on superfrost glass slides (Superfrost Plus; Fisher Scientific, Pittsburgh, PA, USA), facilitated by four equidistant cuts into the peripheral retina. Slides were allowed to air dry before mounting in Vectorshield medium (Vector Laboratories, Peterborough, UK) and applying cover slips. The antibodies used are detailed in Table 1.
Retinal wholemounts were imaged using a Z1 Imager epifluorescent microscope and Axiocam HRc camera (Carl Zeiss, Inc., Thornwood, NY, USA) and RNA binding protein with multiple splicing (RBPMS) $^+$ cells were counted in three 0.33-mm$^2$ regions per retinal quadrant at 1, 2, and 3 mm from the optic nerve head (see Fig. 3C). An individual masked to the treatment group conducted counts manually. The mean number of RGC/image was derived from the 12 images, which made up 2.6% of the total retina (50 mm$^2$) and was used to calculate RGC/mm$^2$ with each group consisting of 10 retinae from five different animals.

**FIGURE 3.** Surviving RBPMS $^+$ RGC count. (A, B) Mean ± SEM number of surviving RBPMS $^+$ RGC 56 days after ic microbead injection (A) or 21 days after laser photocoagulation (B). Counts were conducted in a 0.33-mm$^2$ region of retinal wholemounts, calculated as a composite average of 12 images taken at 1, 2, and 3-mm distances from the optic nerve head, four images per retinal petal (C; scale bar, 1 mm). Asterisks indicate significant difference from injured/untreated at $P < 0.05$. (D) Representative images of RBPMS $^+$ RGC from immunohistochemically stained retinal wholemounts (scale bar: 250 μm). (E) Mean number of surviving RBPMS $^+$ RGC 3 days after purification and culture. Asterisks indicate significant difference at $P < 0.01$. 
Extracellular Vesicle Treatment for Glaucoma

RNA Sequencing

RNAseq was performed by System Biosciences (#CSEQ400A-1) on sEV isolated from BMSC and fibroblasts (as detailed above), three replicates per group. sEV RNA was quantified by bioanalyzer small RNA assay (Agilent, Santa Clara, CA, USA) and libraries constructed and sequenced using Illumina NextSeq instrument with 1 × 75 bp single-end reads at an approximate depth of 10 to 15 million reads per sample. A scaling factor for a given sample was computed as the median of the ratio of its read count for each gene over its geometric mean across all samples. Raw read counts were divided by the factor associated with their samples for normalization. Unlike protein-coding genes/miRNA-seq data analysis in which only uniquely mapped reads are considered, the miRNA pipeline needs to allow multiple mapping of the same read to account for the multiple copies. Thus, normalization was done on the number of read alignments mapped to annotated gene features across samples instead of the number of mapped reads.

The RNAseq data was displayed as a heat map of the log2-fold change between fibroblast and BMSC sEV. A miRNA was considered differentially abundant when the log2-fold change was > 2 or < -2. Using Ingenuity Pathway Analysis software, miRNA upregulated in BMSC sEV as well as their predicted targets were mapped. Predicted targets were only considered if they were experimentally observed findings and the miRNA/miRNA sequences were present in both rat and human.

Statistics

All statistical tests were performed using SPSS 17.0 (IBM SPSS, Inc., Chicago, IL, USA) and data presented as mean ± SEM with graphs constructed using Graphpad Prism 7.01 (Graphpad Prism, La Jolla, CA, USA). The Shapiro-Wilk test was used to ensure all data were normally distributed before parametric testing using a 1-way ANOVA with a Tukey post hoc test. Statistical differences were considered significant at P values < 0.05.

Results

BMSC-Secreted sEV

sEV isolated from human BMSC and fibroblasts were visualized clearly by NanoSight and had a diameter of 100 to 120 nm, as expected for exosomes (Fig. 1B). Very few larger EV were detected and many of those that were detected were likely exosomal aggregates. While NanoSight cannot distinguish sEV from lipoprotein particles, Western blot data showed the absence of ApoA1 and ApoB, markers of lipoproteins, while markers of exosomes/sEV including Syntenin-1 and CD63 were detectable (Supplementary Fig. S2).

Intracameral Microbeads and Laser Photocoagulation of the Trabecular Meshwork Led to Elevations in IOP

Microbeads delivered ic (Group 2) led to a significant rise in IOP from 9.0 ± 0.5 mm Hg (Day 0) to 20.5 ± 2.4 mm Hg (Day 3), which remained high till the end of the experiment (14.2 ± 2.0 mm Hg; Day 56; Fig. 2A). In contrast, IOP in uninjected eyes (Group 1; 11.4 ± 0.7 mm Hg; Day 0) did not change significantly (10.3 ± 0.6 mm Hg; Day 56). The injection of sEV into the vitreous did not significantly (P < 0.05) affect the IOP in laser-photocoagulated eyes.

Intravitreal BMSC sEV Promote Neuroprotection

The intact cohort of Sprague Dawley had an RGC density of 2703.2 ± 73.5 RGC/mm², which was similar to values obtained in other studies. The number of RBPMs' RGC at day 56 in retinal wholemounts decreased significantly in the ic microbead glaucoma model (1468.7 ± 186.3 RGC/mm²) compared with intact eyes (2703.2 ± 73.5 RGC/mm²; Fig. 3A). Monthly and weekly ivit injection of BMSC sEV provided significant neuroprotection of RGC (2113.8 ± 99.0 RGC/mm², 2438.4 ± 123.8 RGC/mm², respectively) but not single injection (1487.5 ± 27.3 RGC/mm²). Monthly and weekly ivit injection of fibroblast sEV did not provide any neuroprotection to RGC (1485.0 ± 216.7 RGC/mm², 1563.4 ± 188.2 RGC/mm², respectively).

The number of RGC at day 21 in retinal wholemounts decreased significantly in the laser photocoagulation glaucoma model (1717.5 ± 173.6 RGC/mm²) compared with intact eyes (2703.2 ± 73.5 RGC/mm²; Fig. 3B). Single and weekly ivit injection of BMSC sEV provided significant neuroprotection of RGC (2416.3 ± 49.6 RGC/mm², 2609.3 ± 66.3 RGC/mm², respectively). This protective effect was partially abolished after ivit injection of sEV isolated from BMSC transfected with siAgo2 (2191.0 ± 23.8 μm) but was still present if sEV isolated from BMSC transfected with siScr were used (2550.2 ± 109.7 μm). Successful knockdown of AGO2 with siAgo2 was confirmed using Western blot (Supplementary Fig. S1). Single and weekly ivit injection of fibroblast sEV did not provide any neuroprotective effect to RGC (1975.6 ± 6 RGC/mm², 1850.6 ± 108.9 RGC/mm², respectively).

BMSC sEV Promote Neuroprotection of RGC Partially Through Direct Mechanisms

Purified RGC die very rapidly, due to the necessary axotomy when culturing along with the lack of present supporting cells. Untreated purified cultures of RGC showed significant death (25.7 ± 1.7 RGC/well) relative to the 5000 RGC plated after 3 days in culture (Fig. 3E). While fibroblast sEV provided little to no neuroprotective effect (60.7 ± 13.0 RGC/well) BMSC sEV elicited significant neuroprotection of (241 ± 17.7 RGC/well).

Intravitreal BMSC sEV Preserve RNFL Thickness/ RGC Axonal Density

The thickness of the RNFL at day 21 decreased significantly in the laser photocogulation glaucoma model (31.8 ± 1.1 μm) compared with intact eyes (47.8 ± 1.8 μm; Fig. 4). Single and weekly ivit injection of BMSC sEV partially prevented degenerative thinning of the RNFL (46.0 ± 1.3 μm; 45.0 ± 1.8 μm, respectively). This protective effect was not present after ivit injection of sEV isolated from BMSC transfected with siAgo2 (32.4 ± 3.2 μm) but was still present if sEV isolated from BMSC transfected with siScr were used (46.8 ± 1.7 μm). Single and weekly ivit injection of fibroblast sEV did not prevent degenerative thinning of the RNFL (33.8 ± 1.5 μm; 34.0 ± 1.5 μm, respectively). There was no significant difference between baseline recordings of all groups as well as compared with intact at day 21 (data not shown).
Intravitreal BMSC sEV Preserve pSTR Amplitude/RGC Function

The amplitude of the pSTR at day 21 decreased significantly in the laser photocoagulation glaucoma model (23.6 ± 2.2 µV) compared with intact eyes (55.5 ± 0.7 µV; Fig. 5). Single and weekly ivit injection of BMSC sEV prevented degenerative loss of the pSTR (39.5 ± 5.1 µV; 44.3 ± 1.7 µV, respectively). This protective effect was not present after ivit injection of sEV isolated from BMSC transfected with siAgo2 (21.8 ± 1.4 µV) but was still present if sEV isolated from BMSC transfected with siScr were used (38.2 ± 2.1 µV). Single and weekly ivit injection of fibroblast sEV did not prevent degenerative loss of the pSTR (26.2 ± 1.1; 25.5 ± 2.5 µm, respectively). There was

**Figure 4.** RNFL thickness measurements of rats. (A) Mean ± SEM RNFL thickness (µm) of rats measured by OCT in animals 21 days after laser photocoagulation. Asterisks indicate significant difference from injured/untreated at P < 0.05. (B) RNFL measurements were taken from around the optic nerve head (green circle; scale bar, 1 mm). (C) Representative OCT images of retina from which the RNFL (partially marked in red) measurements were taken (scale bar: 200 µm).

**Figure 5.** ERG measurements of pSTR. (A) Mean ± SEM amplitude (µV) of pSTR measured by ERG in animals 21 days after laser photocoagulation. Asterisks indicate significant difference from injured/untreated at P < 0.05. (B) Representative traces of pSTR.
FIGURE 6. miRNA abundance heat map profile. A comparison of the miRNA abundance profile in BMSC sEV and fibroblast sEV, displayed as a heat map of the log$_2$-fold change. Red indicates high comparative abundance, whereas green denotes low comparative abundance. Average log$_2$-fold change, an abundance value $>200$ reads per million (Asterisk) as well as $p$ value are given. miRNA with $>2$ or $<-2$ fold change only are shown.
no significant difference between baseline recordings of all groups as well as compared with intact at day 21 (data not shown).

**RNAseq Data**

RNAseq detected 43 miRNA that were overabundant in BMSC sEV compared with fibroblast sEV and 24 miRNA that were overabundant in fibroblast sEV by >2 compared with BMSC sEV (Fig. 6). Using ingenuity pathway analysis, targets of the abundant miRNA were predicted (Fig. 7) and the most enriched/targeted-signaling pathway identified (Table 2).

**DISCUSSION**

The present study demonstrates that sEV derived from BMSC promote significant neuroprotection of RBPMS\^+ RGC in two
separate and distinct models of glaucoma while protecting against RNFL degeneration and pSTR loss. We attributed the protective effect at least partially to the miRNA found within sEV, a finding corroborated by our previous study in an ONG rat model.52 We used two models of glaucoma in this study to minimize the limitations that are present in a single glaucoma model. For example, the laser model suffers from being a short, recoverable rise in IOP whereas the ic microbead model, while being characterized by a persistent rise in IOP, suffers from opacities due to the beads in the anterior chamber, rendering ERG and OCT unreliable. As not every animal responded with elevated ocular hypertension, additional animals were run to ensure each treatment subgroup consisted of five animals/10 eyes.

While MSC are currently undergoing clinical trials to test their efficacy is various ocular diseases,13 their sEV have been demonstrated safe in a recent clinical trial.90 Systemic delivery of UCB-MSC-derived sEV into patients with chronic kidney disease proved not only safe but also, significantly improved kidney function while reducing inflammation. Our previous study showed that sEV integrate into cells within the ganglion cell layer, leading us to speculate that the effect was direct, independent of non-RGC retinal cell mediators. Our current findings demonstrate that in a purified culture, RGC undergo the stereotypical 95% death after 4 days40,41 while treatment with sEV from BMSC, but not fibroblasts, promoted significant RGC neuroprotection. This further confirms that at least part of the neuroprotective effect is mediated through direct sEV-RGC interactions. It should be noted that the RGC are purified based on their expression of Thy-1, a marker expressed in only 80% of RGC yet expressed in cholinergic amacrine cells.42 While 5000 RGC are plated, only approximately 30% attach to the plate.40 Thus, one criticism of the purified RGC culture is it likely does not encompass every RGC subtype.

The present study corroborates our previous findings that showed BMSC sEV-mediated protection of RGC after ONC in the rat. While a recent review highlights the growing trend in ocular exosome/sEV research,43 few studies exist testing sEV as the rat. While a recent review highlights the growing trend in ocular exosome/sEV research,43 few studies exist testing sEV as the rat. While a recent review highlights the growing trend in ocular exosome/sEV research,43 few studies exist testing sEV as the rat. While a recent review highlights the growing trend in ocular exosome/sEV research,43 few studies exist testing sEV as the rat. While a recent review highlights the growing trend in ocular exosome/sEV research,43 few studies exist testing sEV as the rat. While a recent review highlights the growing trend in ocular exosome/sEV research,43 few studies exist testing sEV as the rat.

Taking these results into account, we further confirmed our previous findings in that BMSC sEV were completely absent without a second injection after 1 month, suggesting that BMSC sEV activity drops dramatically after approximately 1 month in the vitreous. This timeframe likely represents both the duration sEV reside in the vitreous and the length of time the miRNA-mediated gene regulation lasts.

AGO2 knockdown-mediated depletion of miRNA partially inhibited the positive effects elicited by sEV evident by the reduced RGC numbers, RNFL thickness, and pSTR amplitude; with this suggestion that miRNA are integral to the mechanism, we performed miRNAseq on BMSC sEV to aid in the potential identification of candidates. We were able to identify candidate miRNA that were more abundant in BMSC sEV in comparison to fibroblast sEV. Several recent studies have analyzed the miRNA content of MSC sEV. The first study identified 11 miRNA present in UCB-MSC-derived sEV via microarray46 while the second study detailed the top 100 most abundant miRNA using a separate method demonstrating high overlap. These miRNA were also detected by our RNaseq, however only MIR-100-5P and MIR-106A-5P were significantly more abundant in BMSC sEV in comparison to fibroblast sEV. A third study performed RNAseq on human ADSC-derived sEV to identify the miRNA potentially responsible for the observed anticancer properties.48 Several of the miRNA that were detected were also identified in the present study including MIR-1246 and MIR-200-5P however, these miRNA were also found in equal or greater abundance within fibroblast sEV, suggesting that they may not be integral to the neuroprotective effects elicited. One miRNA detected was also present in our data, MIR-146-5P, and was more abundant (1.8-log fold change higher) in BMSC sEV compared with fibroblast sEV, as well as being one of the most abundant miRNA. This result has been further confirmed by a separate RNAseq analysis performed on both human BMSC and ADSC as well as demonstrating that abundant miRNA differed between each MSC source.

While abundance of particular BMSC-miRNA in comparison to fibroblasts is critical for the identification of candidates, sEV-mediated delivery of miRNA to the retina can only be effective if the packaged miRNA are not already abundantly present in the injured retina. For example, one recent study analyzed rat retina 7 days after ocular hypertension,19 using microarray to detect overabundant miRNA, whereas a separate study analyzed 16 human retina from cadavers without any retinal pathology, determining the 40 most highly abundant.50 From these studies and the identified miRNA of the present study; MIR-144-5P, MIR-126-5P, and MIR-100-5P were found to be overabundant in BMSC sEV. Because these miRNA are already present in the retina, their contribution following sEV-mediated delivery is likely minimal. In contrast, it has been demonstrated that in the glaucomatous rat retina, several miRNA are downregulated including mir-106b,51 which we found to be abundant in BMSC sEV. It is feasible that downregulation of miRNA has some role in the pathology of glaucoma and delivery of these miRNA via BMSC sEV prevents RGC degeneration. One caveat to the above studies is that miRNA abundance was quantified in total retina as opposed to purified RGC. Currently, no such study exists that provides a detailed analysis of the miRNA present in RGC both before, and after injury.
While we believe that miRNA play an integral role in the sEV-mediated neuroprotection of RGC, it is important to note that miRNA have been found associated with other molecules including protein aggregates and virus particles, both of which could be found within sEV preparation. Because the miRNA associated with these protein aggregates also contains AGO2, it is possible that the diminished therapeutic benefit after AGO2 knockdown is due to these miRNA as well as sEV associated miRNA. Furthermore, after knockdown of AGO2 (siAGO2), sEV still trended toward neuroprotection. As siAGO2 yielded an incomplete (>70%) AGO2 knockdown, it is possible that residual miRNA function was present and responsible for this trend in RGC neuroprotection. Alternatively, non-miRNA components of the BMSC sEV cargo may indeed contribute some beneficial effects and include not only miRNA but approximately 5000 proteins. Recently, BMSC-derived sEV were shown to express an isoform of PDGF referred to as PDGF-D. Because PDGF was been shown to be secreted from BMSC and promote significant RGC neuroprotection, it is possible that the PDGF loaded in sEV elicits similar effects.

In conclusion, BMSC sEV promote neuroprotection and functional preservation of RGC in two rat glaucomatous models. While ivit injection of sEV did not directly affect IOP, their neuroprotective efficacy makes them a good candidate as an adjunctive therapy to IOP-lowering medications, and thus, a potential future treatment for glaucoma.

Acknowledgments
The authors thank Robert Fariss, PhD, for his assistance with laser model, Haohua Qian, PhD, for his assistance with the ERG and OCT, Jennifer Jones, PhD, for her assistance with the NanoSight, Heather Mak, PhD, and Christopher Leung, PhD, for their help with RGC isolation protocol, and Sammy Bennet for his assistance in immunohistochemistry and cell counting. Supported by the Intramural Research Programs of the National Eye Institute (Bethesda, MD, USA).

Disclosure: B. Mead, None; J. Amaral, None; S. Tomarev, None

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