UC Davis
UC Davis Previously Published Works

Title

Protective Effect of Intravitreal Administration of Exosomes Derived from Mesenchymal Stem Cells on Retinal Ischemia

Permalink
https://escholarship.org/uc/item/890344jp

Journal
Current Eye Research, 42(10)

ISSN
0271-3683

Authors
Moisseiev, Elad
Anderson, Johnathon D
Oltjen, Sharon
et al.

Publication Date
2017-10-03

DOI
10.1080/02713683.2017.1319491

Peer reviewed

eScholarship.org Powered by the California Digital Library
University of California

Current Eye Research

ISSN: 0271-3683 (Print) 1460-2202 (Online) Journal homepage: http://www.tandfonline.com/loi/icey20

Protective Effect of Intravitreal Administration of Exosomes Derived from Mesenchymal Stem Cells on Retinal Ischemia

Elad Moisseiev, Johnathon D. Anderson, Sharon Oltjen, Mayank Goswami, Robert J. Zawadzki, Jan A. Nolta & Susanna S. Park

To cite this article: Elad Moisseiev, Johnathon D. Anderson, Sharon Oltjen, Mayank Goswami, Robert J. Zawadzki, Jan A. Nolta & Susanna S. Park (2017) Protective Effect of Intravitreal Administration of Exosomes Derived from Mesenchymal Stem Cells on Retinal Ischemia, Current Eye Research, 42:10, 1358-1367, DOI: 10.1080/02713683.2017.1319491

To link to this article: http://dx.doi.org/10.1080/02713683.2017.1319491

View supplementary material

Published online: 21 Jun 2017.

Submit your article to this journal

Article views: 183

View related articles

View Crossmark data

Citing articles: 1 View citing articles

Full Terms & Conditions of access and use can be found at
http://www.tandfonline.com/action/journalInformation?journalCode=icey20

Download by: [The UC Davis Libraries] **Date:** 05 October 2017, At: 16:12

CURRENT EYE RESEARCH
2017, VOL. 42, NO. 10, 1358–1367
https://doi.org/10.1080/02713683.2017.1319491

Protective Effect of Intravitreal Administration of Exosomes Derived from Mesenchymal Stem Cells on Retinal Ischemia

Elad Moisseiev[a,b], Johnathon D. Anderson[c], Sharon Oltjen[d], Mayank Goswami[e], Robert J. Zawadzki[a,e], Jan A. Nolta[c], and Susanna S. Park[a]

[a]Department of Ophthalmology and Vision Science, University of California Davis Eye Center, Sacramento, CA, USA; [b]Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel; [c]Stem Cell Program, Institute for Regenerative Cures, UC Davis Medical Center, Sacramento, CA, USA; [d]Vitreoretinal Research Laboratory, Department of Ophthalmology and Vision Science, University of California Davis Eye Center, Sacramento, CA, USA; [e]UC Davis RISE Eye-Pod Laboratory, Department of Cell Biology and Human Anatomy, University of California Davis, Davis, CA, USA

ABSTRACT

Purpose: Exosomes derived from human mesenchymal stem cells (hMSCs) cultured under hypoxic conditions contain proteins and growth factors that promote angiogenesis. This study investigated the effect of intravitreal administration of these exosomes on retinal ischemia using a murine model.
Methods: Oxygen-induced retinopathy (OIR) was induced by exposing one-week-old male C57BL/6J mice to 5 days of 75% hyperoxic conditioning, and returning to room air. After hyperoxic conditioning, the right eye of each mouse was injected intravitreally with 1 µl saline or exosomes derived from hMSCs and compared to control mice of the same age raised in room air without OIR injected intravitreally with saline. Two weeks post-injection, fluorescein angiography (FA) and phase-variance optical coherence tomography angiography (pvOCTA) were used to assess retinal perfusion. Retinal thickness was determined by OCT. The extent of retinal neovascularization was quantitated histologically by counting vascular nuclei on the retinal surface.
Results: Among eyes with OIR, intravitreal exosome treatment partially preserved retinal vascular flow *in vivo* and reduced associated retinal thinning; retinal thickness on OCT was 111.1 ± 7.4µm with saline versus 132.1 ± 11.6µm with exosome, $p < 0.001$. Retinal neovascularization among OIR eyes was reduced with exosome treatment when compared to saline-treated eyes (7.75 ± 3.68 versus 2.68 ± 1.35 neovascular nuclei per section, $p < 0.0001$). No immunogenicity or ocular/systemic adverse effect was associated with intravitreal exosome treatment.
Conclusions: Intravitreal administration of exosomes derived from hMSCs was well tolerated without immunosuppression and decreased the severity of retinal ischemia in this murine model. This appealing novel non-cellular therapeutic approach warrants further exploration.

ARTICLE HISTORY
Received 4 January 2017
Revised 5 April 2017
Accepted 7 April 2017

KEYWORDS
Angiogenesis; exosomes; mesenchymal stem cells; oxygen induced retinopathy; retinal ischemia

Introduction

Bone marrow-derived mesenchymal stem cells (MSCs) have tissue healing capabilities and are currently widely explored for numerous therapeutic applications. It has been demonstrated that the effects of MSCs are mediated mostly via paracrine signaling factors to surrounding endogenous cells, rather than direct cell replacement.[1–4]

Exosomes are small (50–150 nm) bi-lipid membrane intracellular vesicles, which are packaged with and transport a variety of proteins and RNAs from their cell of origin to neighboring cells.[5] Exosomes were recently characterized to provide cell-to-cell communication that mediates complex cellular processes, such as antigen cross-presentation, stem cell differentiation and angiogenesis.[6–9]

Recent studies have demonstrated that exosomes derived from MSCs have a protective effect in models of tissue ischemia and reperfusion injury.[9–16] For example, treatment with exosomes derived from MSCs significantly decreased myocardial infarction size in murine models of myocardial ischemia-reperfusion[12,13] and significantly improved blood flow recovery in hind limb ischemia models.[9] A recent comprehensive proteomic analysis of exosomes derived from human MSCs cultured under hypoxic, serum-free conditions revealed that these exosomes express a diverse profile of factors involved in angiogenesis including signaling proteins associated with nuclear factor kappa B (NFkB), platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and fibroblast growth factor (FGF).[17]

Studies have determined that intravitreal injection of MSCs in animal eyes with acute ischemia-reperfusion injury results in preservation of the ganglion cell layer, an effect that could be replicated by intravitreal injection of conditioned media.[18] Since some safety issues have been noted using intravitreal injection of MSCs in some animal models,[19] this study investigated the effect of intravitreal administration of exosomes derived from MSCs as a potential non-cellular therapy for retinal ischemia.

CONTACT Susanna S. Park sscpark@ucdavis.edu Department of Ophthalmology and Vision Science, University of California Davis Eye Center, 4860 Y Street, Suite 2400, Sacramento, CA 95817, USA.

Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/icey.
Supplemental data for this article can be accessed on the publisher's website.

© 2017 Taylor & Francis

Downloaded by [The UC Davis Libraries] at 16:12 05 October 2017

Downloaded by [The UC Davis Libraries] at 16:12 05 October 2017

Methods

Animals

This study protocol was approved by the Institutional Animal Care and Use Committee at the University of California Davis before initiation. The study was conducted according to an approved protocol and in accordance with AAALAC and with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research.

Twelve C57BL/6J mice (Jackson Laboratories; strain 000664) were used in this study. All mice were male and one week of age at the initiation of the study. The mice were maintained with their nursing mothers at the vivarium for the duration of the study until 4 weeks of age. Mice were divided into three groups (see Intravitreal Injections section below), and all mice in all three groups were from the same litter.

Oxygen-induced retinopathy model

A well-established protocol for inducing oxygen-induced retinopathy (OIR) in mice was used to induce retinal ischemia simulating retinopathy of prematurity (ROP).[20] At the age of one week, eight mice were place in a closed chamber under an oxygen concentration of 75% for 5 days. The chamber remained closed during the 5-day hyperoxic exposure period. The hyperoxic condition was monitored continuously using an oxygen sensor (MiniOX I oxygen analyzer; MSA Instrument Division, Pittsburgh, PA), which ensured an oxygen concentration of 75.0%±2.0% for the entire duration. Following the hyperoxic exposure, room air becomes relatively hypoxic to the mice, and by 2 weeks, all eyes develop retinal ischemia and neovascularization.[20] An advantage of this model is that the level of ischemia is quantifiable, based on the count of neovascular cell nuclei on the vitreal surface of the retina on histology[20] (see Tissue Processing and Histology section below for the full details). The remaining four mice were kept at room air for the same 5-day period, as a control group without OIR.

Exosome isolation

Fresh bone marrow from three young non-smoking males was obtained from a commercial vendor (Lonza). MSCs were isolated as previously described and used for exosome isolation at passage 6.[17] Exosomes were isolated from media (OptiMEM) that had been conditioned by MSCs for 48 hours under 1% oxygen tension (serum-free). The conditioned media were precleared of cells and cellular debris via serial centrifugation of the supernatants: a) 500 x g for 10 minutes, b) 2000 x g for 15 minutes. Exosomes were concentrated and washed from the resulting supernatants using tangential flow filtration with a 300 kDa molecular weight cutoff polyethersulfone (PES) membrane (Pall, Port Washington, NY), using a diafiltration wash step with 500 mL of sterile PBS. This step allowed the elimination of cells, cell debris and microvesicles. The resulting exosome concentrated solution was further concentrated using a VivaSpin filtration column with a 300 kDa molecular weight cutoff PES membrane. The resulting concentrated exosome solution was aliquoted at 10 µL and kept at −80°C until used. Vesicle concentration was measured using DC assay (BioRad, Hercules, CA), and size distribution was determined by NanoSight LM10HS (Malvern, Amesbury, MA). This process has previously been described in detail.[17] This isolation protocol highly enriched the isolates for purified exosomes suspended in a PBS buffer.

Intravitreal injections

Intravitreal injections were performed after the 5 days of hyperoxic exposure, when the mice were 12 days old. An intravitreal injection was performed once in the right eye of each mouse. It was performed using a pars planar and transconjunctival approach under isoflurane (2–3% in oxygen) anesthesia. After instilling a drop of 5% betadine solution into the fornix, a sterile 33g needle attached to a Hamilton syringe was used to deliver 1 µl of isolated exosome solution or saline per eye. There were three groups of mice (n = 4 in each group), which were injected as follows: Group 1 included four mice that underwent the OIR induction and were injected with 1 µl saline; Group 2 included four mice that underwent the OIR induction and were injected with exosomes derived from human MSCs (20 µg in 1 µl), and Group 3 included four mice without OIR that were kept at room air at all times and injected with 1 µl saline. Following intravitreal injection, antibiotic eye ointment was applied to the injected eye.

Retinal imaging

Animals were imaged 2 weeks after intravitreal injection. Prior to imaging, all eyes were examined by indirect ophthalmoscopy to determine whether any ocular complications had occurred following the intravitreal injection of exosomes, such as intraocular inflammation, hemorrhage, cataract, retinal detachment or endophthalmitis. A multimodal retinal imaging system specifically designed and built for *in vivo* mouse retinal imaging was used. This system integrates multichannel scanning laser ophthalmoscopy (SLO) and optical coherence tomography (OCT) and allows simultaneous collection of complementary information from the tissue, greatly simplifying data registration and analysis. This system has been described in detail elsewhere.[21] In this study, it was used to perform simultaneous fluorescein angiography (FA) and phase variance OCT angiography (pvOCTA). The pvOCTA detects flow and perfusion in the retinal vasculature and does not visualize non-perfused vessels.

Fluorescein sodium (0.1 ml of 1%) was injected into the tail vein prior to anesthesia and imaging. The mouse retinal imaging was performed under isoflurane (2–3% in oxygen) inhalation anesthesia. A heating pad was used to maintain normal body temperature, and avoid the development of "cold cataracts" during imaging.[22] The head was held rigidly by a "bite-bar" that also served to keep its snout inside the gaseous isoflurane anesthetic delivery tube.

With its customized scanning head, the scanning field of view (FOV) can be up to 50 degrees, while software control allows limiting the scanning to any square subfield of the larger field. With a customized contact lens mounted to the scan head,

the mouse cornea was kept hydrated and clear, greatly facilitating mouse handling during a single imaging session.

Retinal thickness was measured using OCT B-scan images, which were taken at identical locations (horizontal scans through the optic disc). Retinal thickness was measured as the distance between the internal limiting membrane (ILM) and the retinal pigment epithelium (RPE), between 800 points and 1mm temporal from the optic disc margin on the OCT B-scan image. The retinal thickness values were averaged, and the means were compared using an unpaired two-tailed Student's t-test.

Tissue processing and histology

Following imaging, the mice were euthanized by asphyxiation with gaseous CO_2 in a closed chamber, and the right eyes were harvested promptly for histological analysis. The contralateral eye (untreated left eye) from the exosome-treated mice (Group 2) was harvested and fixed as well for analysis.

The eyes were enucleated, fixed in 4% paraformaldehyde, and embedded in paraffin.[23] For orientation in paraffin, the superior region of each eye was marked using tissue dye (The Davidson Marking System, Bradley Products Inc., Bloomington, MN, catalog #1003–6). Sagittal sections were cut using a Leica RM2125RT microtome (Leica, Nussloch, Germany) at 6 microns, placed on SuperFrost Plus microscope slides, and dried overnight at room temperature. Based on the previous orientation of each eye in the paraffin embedding step, a section through the optic disk represented a sagittal section.

Sections underwent standard Hematoxylin-Eosin staining. For each eye, eight sections were carefully viewed at 40X magnification, and neovascular nuclei on the retinal surface were counted. Sections used for counting included four retinal sections from either side of the optic disc, at a distance of 30 to 90 microns from the optic disc, in accordance with the OIR protocol.[20] In each section, neovascular nuclei were identified by their location on the vitreal side of the ILM on the retinal surface. Therefore, a total of eight sections (four sections from each side of the optic nerve) were counted from each eye. The means for each study group were compared using an unpaired two-tailed Student's t-test. Statistical analysis was performed using IBM SPSS Statistics version 21.0.

Slides were viewed and digitized images captured using a Nikon Eclipse E800 and QCapture software (QImaging, Surrey, Canada).

Proteomic data analysis

Proteomic data analysis was performed on data obtained from the analysis of exosomes derived from human MSCs cultured under hypoxic conditions as previously reported.[17] This is a new analysis of previously published data collected from exosomes derived from hMSC as used in this study.[17] Briefly, a multilayered analysis was employed that included clustered network analysis using CytoScape (=) and Ingenuity Pathway Analysis software (Qiagen, Redwood City, CA, USA). The Spike database was used to detect proteins for which there was experimental evidence for physical interactions (*i.e.*, yeast-2-hybrid, coimmunoprecipitation) and was accessed via CytoScape.

Results

Exosomes derived from human MSCs cultured under hypoxic and serum-free conditions were injected intravitreally into murine eyes with OIR to assess the protective effects of this therapy on ischemic retina. Table 1 summarizes the experimental design of the study arms. The eyes with OIR treated with exosomes had three different sets of controls for comparison: 1) eyes without OIR injected with saline, 2) eyes with OIR injected with saline, and 3) eyes with OIR without treatment (contralateral eye of exosome-treated eye with OIR).

In vivo *retinal imaging*

To evaluate the extent of retinal ischemia and neovascularization, the retinal perfusion of the mice was analyzed *in vivo* using simultaneous combined FA and pvOCTA imaging of the retina 2 weeks following intravitreal injection of saline or exosomes. All eyes of mice that underwent OIR induction protocol developed areas of retinal capillary non-perfusion and retinal neovascularization that were apparent on FA and pvOCTA, whereas eyes of mice grown under room air conditions (i.e., without OIR) had normal retinal vascular filling with no areas of retinal non-perfusion or neovascularization (Figure 1). These areas of retinal capillary non-perfusion and retinal neovascularization on FA and pvOCTA were more pronounced in saline-injected eyes with OIR when compared to OIR eyes treated with intravitreal exosome (Figures 1C and F).

Figure 2 illustrates B-scan cross-sectional OCT images of the retina with superimposed pvOCTA signals (red), showing the location of vascular flow relative to the retinal layers. An increased blood flow on the inner surface of the retina was noted in all eyes with OIR in groups 1 and 2 indicative of retinal neovascularization. Retinal thickness was measured using the OCT B-scan images and was found to be 111.1 ± 7.4 μm in Group 1, 132.1 ± 11.6 μm in Group 2, and 205.9 ± 18.8 μm in Group 3. Eyes with OIR (Groups 1 and 2) had significantly thinner retina in comparison with eyes without OIR of mice that had been exposed only to room air (Group 3) ($p < 0.001$ for both). Among eyes with OIR, retinal thickness in the eye treated with saline (Group 1) was thinner than in the eye treated with exosomes (Group 2) ($p < 0.001$).

Histological analysis of retinal neovascularization

To quantitate the level of retinal ischemia, histologic analysis was conducted. By counting the number of neovascular nuclei on the retinal surface in eight sections per eye taken at similar distances from the optic disc, a quantitative measure of the

Table 1. Summary of study groups.

Group	OIR	Eyes	Intravitreal injection	N	Tests performed at 2 weeks following injection
1	Yes	OD	Saline	4	FA, pvOCTA, histology
2	Yes	OD	Exosomes	4	FA, pvOCTA, histology
2	Yes	OS	No	4	Histology only
3	No (room air)	OD	Saline	4	FA, pvOCTA, histology

(OIR = oxygen induced retinopathy; FA = fluorescein angiography; pvOCTA = phase variance optical coherence tomography angiography; OD = right eye; OS = left eye).

Downloaded by [The UC Davis Libraries] at 16:12 05 October 2017

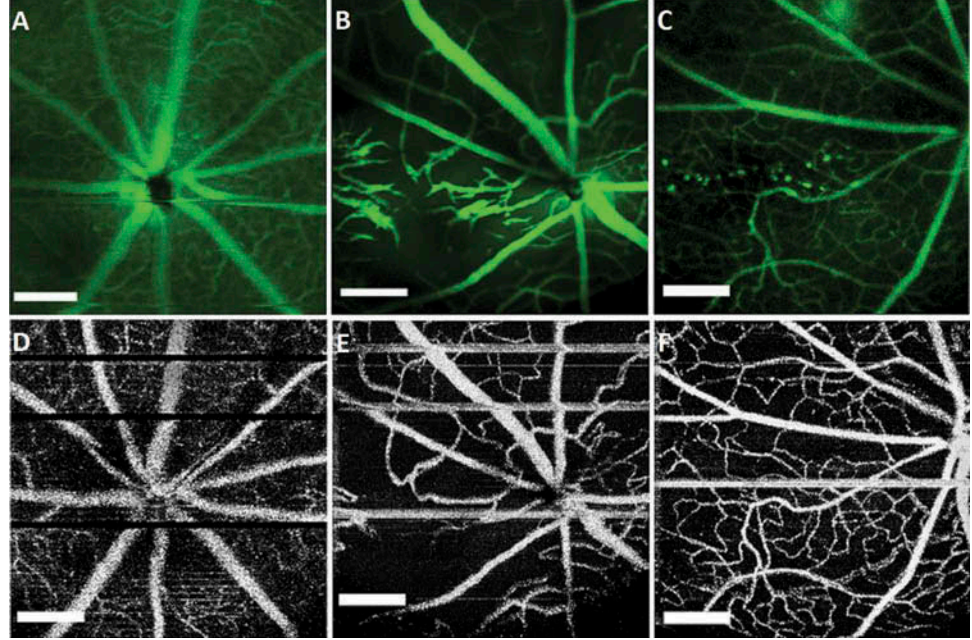


Figure 1. *In vivo* retinal vascular flow imaging of eyes with and without oxygen induced retinopathy (OIR) demonstrating protective effect of intravitreal exosome treatment on retinal ischemia. (A-C) Fluorescein angiogram. Normal retinal perfusion is demonstrated in the eye without OIR (A). In the eyes with OIR, areas of retinal ischemia and neovascularization are seen which are more pronounced in the eye that was treated with intravitreal saline (B) when compared to OIR eyes treated with intravitreal exosomes (C). (D-F) Corresponding phase variance OCT angiography maps of the retinal vascular flow of the same eyes as shown in A (D), B (E) and C (F). Normal retinal capillary perfusion is demonstrated in the eye without OIR (D). The eyes with OIR show marked retinal capillary non-perfusion which is more pronounced in the eye that was treated with intravitreal saline (E) when compared to the eye treated with intravitreal exosomes (F). The horizontal lines are motion artifacts. The length of the horizontal bar scale at the lower left corner of each image represents 0.17 mm.

Downloaded by [The UC Davis Libraries] at 16:12 05 October 2017

degree of retinal neovascularization was obtained for each study eye. An example of retinal neovascularization on the retinal surface, that is, the vitreal side of the ILM is presented in Figure 3.

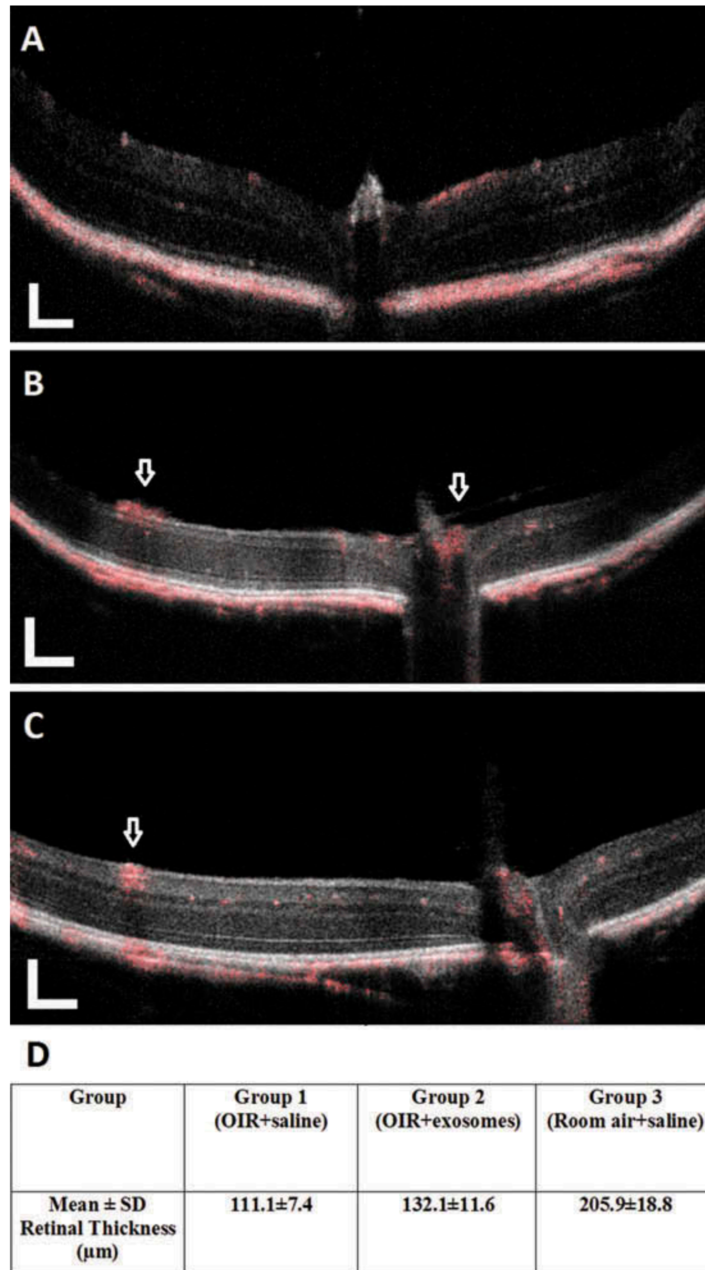
In Group 1, OIR eyes treated with saline, there was an average of 7.75 ± 3.68 neovascular nuclei per section. In Group 2, OIR eyes treated with exosomes, there was an average of 2.68 ± 1.35 neovascular nuclei per section. In the untreated fellow eyes of mice from Group 2, there was an average of 7.0 ± 2.48 neovascular nuclei per section. In eyes without OIR (Group 3), there was an average of 0.12 ± 0.33 neovascular nuclei per section. All eyes from mice exposed to OIR induction had significantly higher neovascular nuclei counts than Group 3 eyes without OIR ($p < 0.0001$ for all). There was no difference between Group 1 eyes (OIR eyes treated with saline) and the untreated fellow eyes of mice from Group 2 (untreated OIR eyes) ($p = 0.35$). In Group 2 eyes treated with exosomes, the neovascular nuclei counts were significantly lower than saline treated Group 1 eyes with OIR and the untreated fellow eyes with OIR in Group 2 ($p < 0.0001$ for both). These results are presented graphically in Figure 3D. We also note that no signs of ocular inflammation were noted on examination and histological analysis of the eyes injected with exosomes.

hMSCs-derived exosome proteomic analysis

To assess factors contained within exosomes derived from human bone marrow-derived MSCs that mediate their protective affects, we further analyzed data obtained previously using a novel, unbiased proteomics method, high-resolution isoelectric focusing liquid chromatography couple tandem mass spectrometry (HiRIEF LC-MS/MS). This new analysis was performed on previously published data collected from exosomes derived from hMSC as used in this study.[17] A total of 1927 proteins were identified in each exosome sample generated from MSCs derived from three different human donors (see Table S1, Supplemental Digital Content 1.We previously reported that the exosomes expressed 92 of the top 100 most identified exosomal marker proteins from the ExoCarta database in our exosome samples (see Table S2, Supplemental Digital Content 2, and Figure S1, Supplemental Digital Content 3). We also previously reported the vascular-protective proteins identified in MSC exosomes.[17]

Here, we present new analysis of the proteomic data demonstrating that exosomes derived from human MSCs are packaged with numerous pro-survival-associated proteins from the cAMP response element-binding protein (CREB) pathway using Ingenuity Pathway Analysis (Qiagen) (Figure 4). Clustered network analysis of protein–protein interaction networks (CytoScape, Spike database) determined clustering of proteins associated with pro-survival heat shock protein (HSP) pathways: HSPA1A, HSPA4, HSPA5, HSPA8, HSPA9, HSP90AA1, HSPB90, HSPBP1, HSPD1, HSPG2, HSPH1 (Figure 5). These data collectively demonstrate that exosomes derived from human MSCs contain numerous proteins associated with pro-survival signaling cascades. The delivery of such prosurvival proteins to retinal tissues serves as an

Downloaded by [The UC Davis Libraries] at 16:12 05 October 2017



Group	Group 1 (OIR+saline)	Group 2 (OIR+exosomes)	Group 3 (Room air+saline)
Mean ± SD Retinal Thickness (µm)	111.1±7.4	132.1±11.6	205.9±18.8

Figure 2. Composite OCT and phase-variance OCT angiography B-scan images of study eyes showing differences in retinal thickness in the three study groups. The retina is notably thicker in the eye without OIR (A) when compared to the eye with OIR treated with saline (B) or exosomes (C). Red indicates vascular flow, and retinal neovascularization is demonstrated by red on the retinal surface in eyes with OIR (B,C) (hollow arrows). The length of the vertical and horizontal arm of the L-shaped scale at the lower left corner of each image represents 100 µm.(D) Mean ± SD of retinal thickness measurements in each study group. Both OIR groups (Groups 1 and 2) had significantly reduced retinal thickness compared with the control group (Group 3); the retinal thickness was significantly reduced in saline-treated eyes (Group 1) in comparison with exosome-treated eyes (Group 2) ($p < 0.001$ for all).

additional potential mechanism by which MSC exosomes may mediate their protective effects in the OIR model of retinopathy.

Discussion

In this study, we used a murine model of OIR to study the effect of intravitreal administration of exosomes derived from human bone marrow MSCs on retinal ischemia. This model was used as it is a well-established, validated and quantifiable model for retinal ischemia.[20,24] We used a novel three-dimensional *in vivo* retinal imaging system specifically designed for murine eyes, that is, pvOCTA, as well as the traditional FA to evaluate retinal perfusion *in vivo*. Furthermore, histologic analysis was used to evaluate the effect of intravitreal administration of exosomes from human bone marrow MSCs in this model of retinal ischemia.

Both *in vivo* retinal imaging techniques, pvOCTA and FA, demonstrated that the degree of retinal ischemia and the development of retinal neovascularization in eyes exposed to the OIR induction were reduced in eyes treated with intravitreal exosomes (Group 2) compared to saline treated controls (Group 1) (Figure 1). The newer pvOCTA allows higher resolution three-dimensional imaging of the retinal

Downloaded by [The UC Davis Libraries] at 16:12 05 October 2017

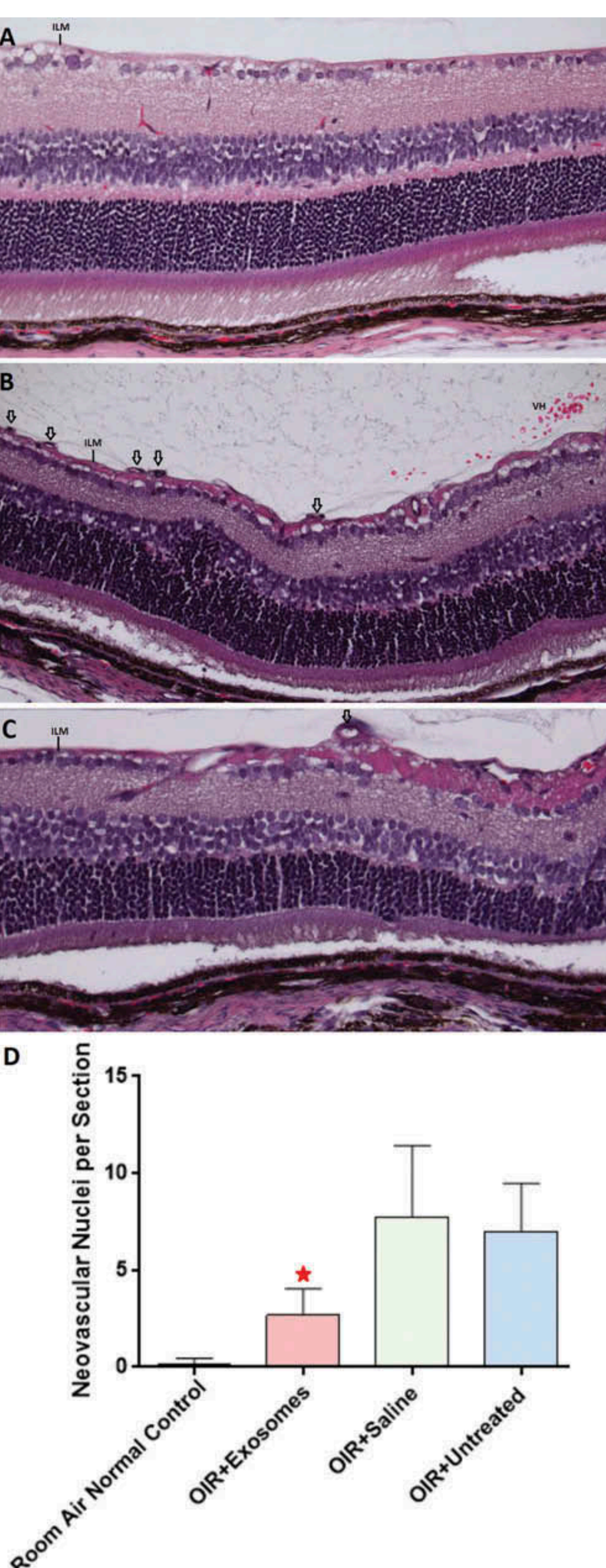


Figure 3. Histologic retinal section with Hematoxylin-Eosin staining demonstrating retinal neovascularization on the retinal surface. (A) A cross section of the retina from an eye without OIR shows no neovascular nuclei on the retinal surface. (B) A cross section of the retina from an eye with OIR treated with saline showing several foci of retinal neovascularizations extending to the vitreal side of the ILM (hollow arrows), as well as some red blood cells in the vitreous indicating associated vitreous hemorrhage. (C). A cross section of the retina from an eye with OIR treated with exosomes showing an area of retinal neovascularization (hollow arrow). There were significantly less neovascular nuclei on the vitreal side of the ILM in these eyes compared to control OIR eyes treated with saline. [ILM = internal limiting membrane; VH = vitreous hemorrhage). The separation of the retina from the RPE is an artifact of fixation. (D). Comparison of mean±SD neovascular nuclei counts of the eyes included in the study. The values represent means obtained from four eyes per group with eight sections per eye. Eyes treated with exosomes (group 2) had significantly lower neovascular nuclei counts than eyes treated with saline (Group 1), as well as their fellow untreated eyes ($P < 0.001$ for both, indicated by a red star).

Downloaded by [The UC Davis Libraries] at 16:12 05 October 2017

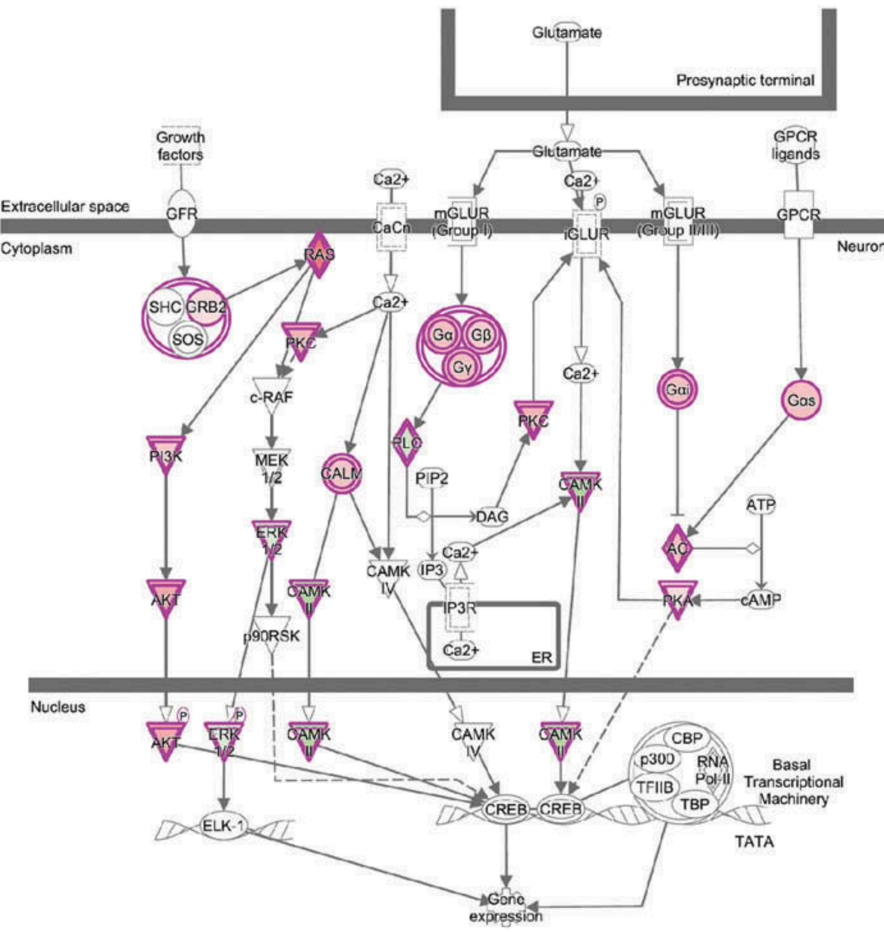


Figure 4. Ingenuity Pathway Analysis demonstrated exosomes derived from hMSCs are packaged with CREB signaling proteins that have been shown to mediate survival of neurons and other cell types. Colored nodes indicate detection in the exosomes, white nodes indicates not detected.

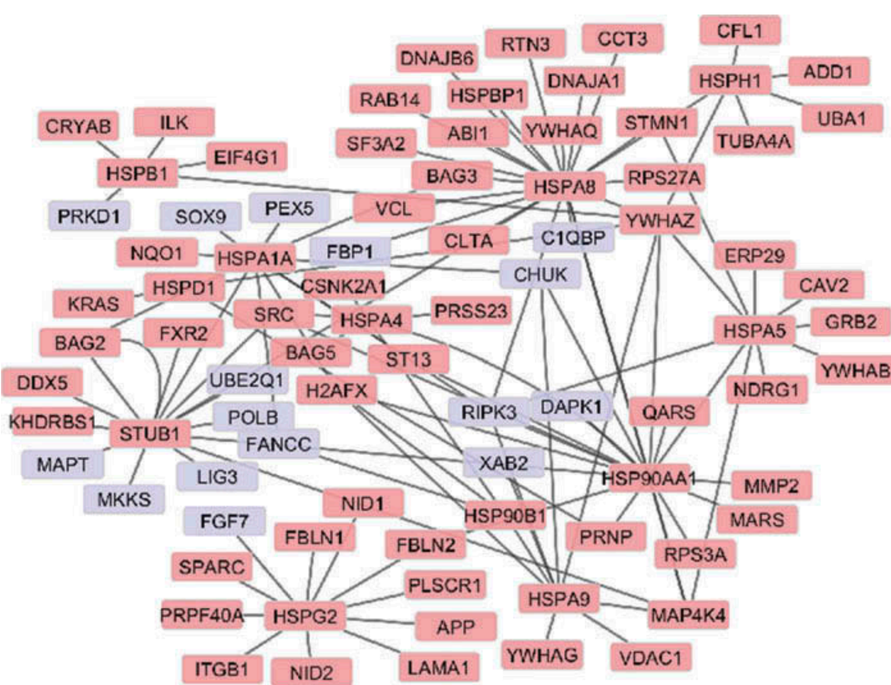


Figure 5. Clustered network analysis of protein–protein interaction networks using CytoScape and the Spike database demonstrated presence of HSP associated pathway proteins. Red nodes = detected in exosomes, blue nodes = not detected in exosomes. Edges indicate protein-protein interactions as demonstrated by experimental evidence (e.g., co-immunoprecipitation, yeast 2-hybrid).

Downloaded by [The UC Davis Libraries] at 16:12 05 October 2017

vasculature.[25] The change in retinal perfusion was appreciated at all levels of the retinal vasculature using pvOCTA. The degree of retinal thinning associated with the induced retinal ischemia in eyes with OIR was less pronounced in eyes treated with exosomes (Group 2) when compared to saline treated controls (Group 1) (132.1 ± 11.6 µm vs. 111.1 ± 7.4 µm, respectively, $p < 0.001$). These results indicate that although intravitreal injection of exosomes from human MSCs did not completely prevent retinal ischemia and neovascularization in the OIR model, the treatment significantly attenuated the severity of OIR both qualitatively and quantitatively.

The histologic analysis of eyes in this study further supports the *in vivo* retinal imaging findings that intravitreal injection of exosomes from human MSCs significantly reduced the degree of retinal ischemia and neovascularization associated with OIR. While eyes with OIR treated with saline (Group 1) had similar mean neovascular nuclei counts per eye as the fellow untreated eyes with OIR, eyes with OIR treated with exosomes (Group 2) has significantly lower mean neovascular nuclei counts. This is consistent with the observed reduced level of retinal neovascularization visualized using *in vivo* retinal vascular imaging in these mice. Another important point is that eyes treated with exosomes showed no signs of ocular inflammation on examination and histological analysis.

Murine model of OIR is a well-established preclinical model for quantitating retinal ischemia and neovascularization.[20] However, it is important to point out that some variability in the development of OIR has been reported even among mice of the same strain. In our study, the OIR was induced in the same strain of mice from the same vendor at the same time to minimize any variability between study groups. The number of mice per group was limited by the size of the hyperoxic chamber and litter. The time course of development of retinal neovascularization is reported to peak at postnatal day 17 to 21 (i.e., 1 week after hyperoxic conditioning) with evidence of tissue repair thereafter.[20] Due to logistical issues, our study evaluated mice on postnatal day 26 which may have attenuated the retinal vascular changes associated with OIR. Although this time point may not have been ideal, a statistically significant difference in retina neovascularization was noted between the study groups. The potential advantage of waiting a few days longer till day 26 to compare study groups is that the neuroprotective effects of exosome treatment on retinal thickness may be appreciated more readily.

The effect of exosomes on their target tissues is variable because exosomes secreted from different cell types or under different conditions may vary in their content. Exosomes derived from retinal astroglial cells suppress choroidal neovascularization, while exosomes from retinal pigment epithelial cells have shown pro-angiogenic effects when administered intravenously and via periocular injections.[26,27] With RPE cells, oxidative stress can cause the cells to increase exosome secretion. These exosomes from RPE cells contain higher concentrations of pro-angiogenic signaling proteins in response to oxidative stress.[27,28] Similarly, in our study, hypoxic stress was used to increase exosome production of angiogenic and pro-cell survival factors in cultured MSCs from human bone marrow.[17,29]

The pro-angiogenic profile of the exosomes derived from human MSCs under hypoxic conditions has been reported previously.[17] In this study, we conducted additional proteomic data analysis focused on pro-survival pathways. Our results indicate that these exosomes from human MSCs also contain pro-survival-associated proteins from both the cAMP response element-binding protein (CREB) pathway and shock protein (HSP) pathways. Both of these pathways have been previously shown to be affected by retinal ischemia, directly and as a compensatory response of the retina to ischemia.[30–35] Therefore, the prosurvival proteins elucidated in the present study may help explain MSC exosomes ability to limit retinal degradation induced by the OIR model. Based on these findings, exosomes from human MSCs may have multiple mechanisms of actions for their observed protective effect on retinal ischemia. Numerous angiogenesis and survival pathways in retinal issue may be enhanced by MSC exosome treatment.

The current study explored a new route of exosome administration for retinal ischemia treatment via intravitreal injection. We demonstrated that this route of exosome administration was well tolerated during the two-week duration of this study. The therapy was effective without concurrent use of systemic immunosuppression although the exosomes were derived from human cells and used in mouse eyes, and no ocular inflammation was detected. Furthermore, our study showed that exosomes derived from human MSCs cultured under hypoxic, serum-free conditions were effective in reducing the severity of retinal ischemia in mice with OIR.

Our findings parallel a recent report by Mead and Tomarev who showed that intravitreal injection of exosomes from hMSCs resulted in preservation of ganglion cells in a model of optic nerve injury.[36] They noted that the content of the exosomes was localized in the inner retina, the target tissue. In this study of optic nerve injury by Mead and Tomarev, intravitreal injection of exosomes from fibroblasts was used as a control since the investigators noted no effect of exosomes from fibroblasts on ganglion cell degeneration. In our study, saline was injected in control eyes since exosomes from other cells may contain factors that potentially affect retinal ischemia and neovascularization.

Intravitreal administration of MSCs has been shown to have a protective effect in animal models of retinal ischemia-reperfusion injury. The effect could be simulated with conditioned media.[18,37] Since there are some safety concerns about intravitreal administration of MSCs or conditioned media, the use of exosomes isolated from conditioned media is a potential safe alternative to cell therapy. Since exosomes derived from MSCs have demonstrated efficacy in non-ocular models of tissue ischemia,[9–16] it is reasonable to hypothesize that exosomes from MSCs will have a similarly positive effect on retinal ischemia. Our study results support this hypothesis.

In summary, our study findings show that intravitreal injection of exosomes from human MSCs is well-tolerated and can have a protective therapeutic effect on retinal ischemia. No immunogenicity was detected despite the fact that exosomes from human cells were administered to immunocompetent mice. Intravitreal injection of exosomes from stem cells may be a novel non-cellular approach to achieve efficacy

and benefits of stem cell therapy without exposing the recipient to potential safety concerns associated with cell therapy, such as cellular proliferation or immune rejection. Further studies will need to be performed to determine whether the beneficial effect of these exosomes can be observed in other models of retinal disease. Additional long-term safety information and dose-response assessments are needed before this therapy can be translated to clinical applications. Exosomes derived from human MSCs can be used in an allogeneic manner, since they are immune privileged. In addition, exosomes may hold other advantages beyond the use of MSCs for clinical applications as exosomes may provide a more concentrated source of tissue healing factors with off-the-shelf delivery capabilities. This is a very appealing novel non-cellular therapeutic approach that warrants further exploration in ophthalmology.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper..

Funding

This study was supported in part by the University of California, Davis Eye Center Retina Research Fund. JAN is supported by the California Institute for Regenerative Medicine and an NIH Common fund transformative research projects grant 1R01GM099688. JDA is supported by NSF GRFP 2011116000, NIH T32-GM008799, NSF GROW 201111600, and NIH T32-HL086350. The *in vivo* retinal imaging of mice was conducted at the UC Davis RISE Eye-Pod laboratory supported by the UC Davis Research Investments in Science and Engineering (RISE) initiative, UC Davis NEI Vision Core Grant EY 012576 and NSF I/UCRC CBSS Grant. This work was presented in part as a paper presentation at the Retina Society meeting as recipient of the Retina Society Fellow Research Award (EM), September 15, 2016, San Diego, CA.

References

1. Meyerrose T, Olson S, Pontow S, Kalomoiris S, Jung Y, Annett G, et al. Mesenchymal stem cells for the sustained in vivo delivery of bioactive factors. Adv Drug Deliv Rev 2010;62:1167–1174.
2. Gnecchi M, He H, Liang OD, Melo LG, Morello F, Mu H, et al. Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. Nat Med 2005;11:367–368.
3. Lee JW, Fang X, Krasnodembskaya A, Howard JP, Matthay MA. Concise review: mesenchymal stem cells for acute lung injury: role of paracrine soluble factors. Stem Cells 2011;29:913–919.
4. Rose RA, Jiang H, Wang X, Helke S, Tsoporis JN, Gong N, et al. Bone marrow-derived mesenchymal stromal cells express cardiac-specific markers, retain the stromal phenotype, and do not become functional cardiomyocytes in vitro. Stem Cells 2008;26:2884–2892.
5. Andaloussi EL. S, Mäger I, Breakefield XO, Wood MJ. Extracellular vesicles: biology and emerging therapeutic opportunities. Nat Rev Drug Discov 2013;12:347–357.
6. Théry C, Ostrowski M, Segura E. Membrane vesicles as conveyors of immune responses. Nat Rev Immunol 2009;9:581–593.
7. Zhang B, Yin Y, Lai RC, Tan SS, Choo AB, Lim SK. Mesenchymal stem cells secrete immunologically active exosomes. Stem Cells Dev 2014;23:1233–1244.
8. Lee HK, Finniss S, Cazacu S, Xiang C, Brodie C. Mesenchymal stem cells deliver exogenous miRNAs to neural cells and induce their differentiation and glutamate transporter expression. Stem Cells Dev 2014;23:2851–2861.
9. Hu GW, Li Q, Niu X, Hu B, Liu J, Zhou SM, et al. Exosomes secreted by human-induced pluripotent stem cell-derived mesenchymal stem cells attenuate limb ischemia by promoting angiogenesis in mice. Stem Cell Res Ther 2015;6:10.
10. Bian S, Zhang L, Duan L, Wang X, Min Y, Yu H. Extracellular vesicles derived from human bone marrow mesenchymal stem cells promote angiogenesis in a rat myocardial infarction model. J Mol Med 2014;92:387–397.
11. Zhang B, Wang M, Gong A, Zhang X, Wu X, Zhu Y, et al. HucMSC-exosome mediated-Wnt4 signaling is required for cutaneous wound healing. Stem Cells 2015;33:2158–2168.
12. Lai RC, Arslan F, Lee MM, Sze NS, Choo A, Chen TS, et al. Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. Stem Cell Res 2010;4:214–222.
13. Arslan F, Lai RC, Smeets MB, Akeroyd L, Choo A, Aguor EN, et al. Mesenchymal stem cell-derived exosomes increase ATP levels, decrease oxidative stress and activate PI3K/Akt pathway to enhance myocardial viability and prevent adverse remodeling after myocardial ischemia/reperfusion injury. Stem Cell Res 2013;10:301–312.
14. Teng X, Chen L, Chen W, Yang J, Yang Z, Shen Z. Mesenchymal stem cell-derived exosomes improve the microenvironment of infarcted myocardium contributing to angiogenesis and anti-Inflammation. Cell Physiol Biochem 2015;37:2415–2424.
15. Doeppner TR, Herz J, Görgens A, Schlechter J, Ludwig AK, Radtke S, et al. Extracellular vesicles improve post-stroke neuroregeneration and prevent postischemic immunosuppression. Stem Cells Transl Med 2015;4:1131–1143.
16. Wang R, Lin M, Li L, Li L, Qi G, Rong R, et al. Bone marrow mesenchymal stem cell-derived exosome protects kidney against ischemia reperfusion injury in rats. Zhonghua Yi Xue Za Zhi 2014;94:3298–3303.
17. Anderson JD, Johansson HJ, Graham CS, Vesterlund M, Pham MT, Bramlett CS, et al. Comprehensive proteomic analysis of mesenchymal stem cell exosomes reveals modulation of angiogenesis via nuclear factor-kappab signaling. Stem Cells 2016;34:601–613.
18. Dreixler JC, Poston JN, Balyasnikova I, Shaikh AR, Tupper KY, Conway S, et al. Delayed administration of bone marrow mesenchymal stem cell conditioned medium significantly improves outcome after retinal ischemia in rats. Invest Ophthalmol Vis Sci 2014;55:3785–3796.
19. Park SS. Cell therapy applications for retinal vascular diseases: diabetic retinopathy and retinal vein occlusion. Invest Ophthalmol Vis Sci 2016;57:ORSF.
20. Smith LE, Wesolowski E, McLellan A, Kostyk SK, D'Amato R, Sullivan R, et al. Oxygen-induced retinopathy in the mouse. Invest Ophthalmol Vis Sci 1994;35:101–111.
21. Zhang P, Zam A, Jian Y, Wang X, Li Y, Lam KS, et al. In vivo wide-field multispectral scanning laser ophthalmoscopy–optical coherence tomography mouse retinal imager: longitudinal imaging of ganglion cells, microglia, and Müller glia, and mapping of the mouse retinal and choroidal vasculature. J Biomedical Optics 2015;12:126005.
22. Bermudez MA, Vicente AF, Romero MC, Arcos MD, Abalo JM, Gonzalez F. Time course of cold cataract development in anesthetized mice. Curr Eye Res 2011;36:278–284.
23. Smit-McBride Z, Forward KI, Nguyen AT, Bordbari MH, Oltjen SL, Hjelmeland LM. Age-dependent increase in miRNA-34a expression in the posterior pole of the mouse eye. Mol Vis 2014;20:1569–1578.
24. Grossniklaus HE, Kang SJ, Berglin L. Animal models of choroidal and retinal neovascularization. Prog Retin Eye Res 2010;29:500–519.
25. Schwartz DM, Fingler J, Kim DY, Zawadzki RJ, Morse LS, Park SS, et al. Phase-variance optical coherence tomography: a technique for noninvasive angiography. Ophthalmology 2014;121:180–187.
26. Hajrasouliha AR, Jiang G, Lu Q, Lu H, Kaplan HJ, Zhang HG, et al. Exosomes from retinal astrocytes contain antiangiogenic components that inhibit laser-induced choroidal neovascularization. J Biol Chem 2013;288:28058–28067.

Downloaded by [The UC Davis Libraries] at 16:12 05 October 2017

27. Atienzar-Aroca S, Flores-Bellver M, Serrano-Heras G, Martinez-Gil N, Barcia JM, Aparicio S, et al. Oxidative stress in retinal pigment epithelium cells increases exosome secretion and promotes angiogenesis in endothelial cells. J Cell Mol Med 2016. [ahead of print].
28. Biasutto L, Chiechi A, Couch R, Liotta LA, Espina V. Retinal pigment epithelium (RPE) exosomes contain signaling phosphoproteins affected by oxidative stress. Exp Cell Res 2013;319:2113–2123.
29. Yang J, Gao F, Zhang Y, Liu Y, Zhang D. Buyang Huanwu Decoction (BYHWD) enhances angiogenic effect of mesenchymal stem cell by upregulating VEGF expression after focal cerebral ischemia. J Mol Neurosci 2015;56:898–906.
30. Guo XJ, Tian XS, Ruan Z, Chen YT, Wu L, Gong Q, et al. Dysregulation of neurotrophic and inflammatory systems accompanied by decreased CREB signaling in ischemic rat retina. Exp Eye Res 2014;125:156–163.
31. Joachim SC, Wax MB, Boehm N, Dirk DR, Pfeiffer N, Grus FH. Upregulation of antibody response to heat shock proteins and tissue antigens in an ocular ischemia model. Invest Ophthalmol Vis Sci 2011;52:3468–3474.
32. Biermann J, Lagrèze WA, Dimitriu C, Stoykow C, Goebel U. Preconditioning with inhalative carbon monoxide protects rat retinal ganglion cells from ischemia/reperfusion injury. Invest Ophthalmol Vis Sci 2010;51:3784–3791.
33. Windisch BK, LeVatte TL, Archibald ML, Chauhan BC. Induction of heat shock proteins 27 and 72 in retinal ganglion cells after acute pressure-induced ischaemia. Clin Experiment Ophthalmol 2009;37:299–307.
34. Li Y, Roth S, Laser M, Ma JX, Crosson CE. Retinal preconditioning and the induction of heat-shock protein 27. Invest Ophthalmol Vis Sci 2003;44:1299–1304.
35. Lewden O, Garcher C, Assem M, Morales C, Rochette L, Bron AM. Changes of the inducible heat shock protein 70 mRNA level in rat retina after ischemia and reperfusion. Ophthalmic Res 1998;30:291–294.
36. Mead B, Tomarev S. Bone marrow-derived mesenchymal stem cells-derived exosomes promote survival of retinal ganglion cells through miRNA-dependent mechanisms. Stem Cells Transl Med 2017. doi:10.1002/sctm.12056.
37. Li N, Li X, Yuan J. Effects of bone-marrow mesenchymal stem cells transplanted into the vitreous cavity of rat injured by ischemia/reperfusion. Graefes Arch Clin Exp Ophthalmol 2009;247:503–514.

Downloaded by [The UC Davis Libraries] at 16:12 05 October 2017