

Adipose-Derived Stem Cells From Diabetic Mice Show Impaired Vascular Stabilization in a Murine Model of Diabetic Retinopathy

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ABSTRACT

Diabetic retinopathy is characterized by progressive vascular dropout with subsequent vision loss. We have recently shown that an intravitreal injection of adipose-derived stem cells (ASCs) can stabilize the retinal microvasculature, enabling repair and regeneration of damaged capillary beds in vivo. Because an understanding of ASC status from healthy versus diseased donors will be important as autologous cellular therapies are developed for unmet clinical needs, we took advantage of the hyperglycemic Akimba mouse as a preclinical in vivo model of diabetic retinopathy in an effort aimed at evaluating therapeutic efficacy of adipose-derived stem cells (mASCs) derived either from healthy, nondiabetic or from diabetic mice. To these ends, Akimba mice received intravitreal injections of media conditioned by mASCs or mASCs themselves, subsequent to development of substantial retinal capillary dropout. mASCs from healthy mice were more effective than diabetic mASCs in protecting the diabetic retina from further vascular dropout. Engrafted ASCs were found to preferentially associate with the retinal vasculature. Conditioned medium was unable to recapitulate the vasoprotection seen with injected ASCs. In vitro diabetic ASCs showed decreased proliferation and increased apoptosis compared with healthy mASCs. Diabetic ASCs also secreted less vasoprotective factors than healthy mASCs, as determined by high-throughput enzymelinked immunosorbent assay. Our findings suggest that diabetic ASCs are functionally impaired compared with healthy ASCs and support the utility of an allogeneic injection of ASCs versus autologous or conditioned media approaches in the treatment of diabetic retinopathy. STEM CELLS Translational Medicine 2015;4:459–467

SIGNIFICANCE

This work addresses the therapeutic potential of stem cells that are obtained from diabetic donors, and the clinical focus of our work is diabetic retinopathy. This work examined how injected murine adiposederived stem cells (ASCs) affect the retinal microvasculature in a mouse model of diabetic retinopathy. Results show that ASCs obtained from healthy mice secrete angiogenic growth factors and promote retinal vascular stability when they are injected intravitreally. The findings further suggest that ASCs obtained from diabetic mice have a diminished ability to support the retinal vasculature in this mouse model of retinal vasculopathy.

INTRODUCTION

The therapeutic potential of adipose-derived stem cells (ASCs) has been demonstrated with success in several applications, including myocardial infarction, diabetic wound healing, and neurodegenerative disorders [1–3]. Studies comparing the efficacy of ASCs from diabetic and nondiabetic sources have focused on their application in diabetic ulcers [4] and hind limb ischemia [5]. Both of these studies found that mouse ASCs (mASCs) derived from diabetic sources have impaired treatment efficacy relative to their nondiabetic counterparts. Stem cells from different sources, such as bone marrow, have also shown functional impairment when derived from diabetic mice in the treatment of cardiovascular disease [1, 6].

We have recently shown that intravitreal injection of ASCs stabilizes the retinal microvasculature and encourages regeneration of damaged capillary beds in several mouse models of retinal vasculopathy [7]. ASCs are desirable because of their relative ease of harvest from accessible fat

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http://dx.doi.org/ 10.5966/sctm.2014-0108 depots, as well as their potential for allogeneic or even autologous treatment [8]. However, only a few studies have examined whether ASCs obtained from diabetic donors are negatively impacted by the disease; in turn, negative outcomes here would have implications for the feasibility of autologous cell-based therapies [9]. To date, no studies have assessed the functional impact of diabetes on ASCs for the treatment of diabetic retinopathy.

Diabetes profoundly impacts the microvasculature in nearly every tissue. Diabetic retinopathy results in retinal capillary dropout, vessel leakage, and pathological neovascularization, leading to severe and irreversible vision loss. Current surgical and pharmacologic treatments are only effective at managing complications of diabetic retinopathy but do not repair existing damage [10]. Laser photocoagulation is the current treatment standard for proliferative diabetic retinopathy and operates on the principle of cauterizing hypoxic retinal tissue [11]. Although effective at stemming the progression of retinopathy, this procedure damages peripheral and night vision, often requires repeated treatments, and only prevents visual deterioration in half of cases [10-12]. Anti-vascular endothelial growth factor (VEGF) therapy has been increasingly used alone or in combination with laser therapy, with improvements in vision loss caused by diabetic macular edema [13]. However, anti-VEGF therapy requires frequent intravitreal injections for several years with potential complications and does not reverse the underlying pathology [10]. A lasting, nondestructive treatment for diabetic retinopathy is clearly needed.

Using the Akimba mouse model of diabetic retinopathy, we have probed the differences in treatment efficacy and function of mASCs derived from healthy versus diabetic mice. The Akimba is a cross between the hyperglycemic Akita mouse, which carries a dominant-negative mutation in its *insulin-2* gene, and the Kimba mouse, which transiently overexpresses human VEGF in retinal photoreceptor cells [14, 15]. The combination of these modifications creates a mouse whose retina exhibits hallmarks of proliferative diabetic retinopathy in humans with retinal edema, aberrant neovascularization, and progressive vascular dropout over time [15].

The primary goal of this study was to determine whether mASCs from healthy, nondiabetic mice are more effective than diabetic mASCs from Akimba mice in preventing progressive vascular damage and dropout in the diabetic retina. We also sought to investigate potential functional differences between healthy and diabetic mASCs, both in vivo with respect to their abilities to associate with and affect the retinal vasculature and in vitro with respect to their proliferative capacities, apoptosis rates, metabolic functions, and abilities to secrete soluble factors that promote vascular stability. We were subsequently interested in determining whether a single intravitreal injection of healthy or diabetic ASC-conditioned media was equivalent to injecting either healthy or diabetic hASCs in terms of preventing vascular dropout in the diseased Akimba retina. Our functional analysis of healthy versus diabetic mASCs in an established in vivo model of retinal vascular damage may have implications for the future ocular use of autologous ASCs from diabetic patients. By evaluating different mechanisms and cellular behaviors in both healthy and diabetic mASCs, our study contributes to understanding how ASCs might confer a vasoprotective effect in settings of progressive vascular damage and suggests strategies for enhancing their therapeutic capabilities.

MATERIALS AND METHODS

mASC Harvest and Culture

Isolation of the stromal vascular fraction from the epididymal fat pad and culture of mASCs was performed as detailed by Zuk et al. [8]. Briefly, fat pads were harvested from 9-week-old Akimba (Ins2^{Akita}VEGF^{+/-}) mice and nondiabetic Kimba (VEGF^{+/-}) littermates and then digested in collagenase-containing digestion buffer (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich. com) for 1 hour at 37°C. The resulting mixture was filtered through 200- μ m mesh (Corning Enterprises, Corning, NY, http://www.corning.com) to exclude any undigested tissue. The filtrate was centrifuged, and the pellet was resuspended in phosphate-buffered saline (PBS) and incubated with red blood cell lysis buffer (Sigma-Aldrich) for 5 minutes at room temperature. The cell suspension was then sterile-filtered through 40- μ m mesh and plated on sterile culture plates (Corning Enterprises). Cells harvested from Akimba mice are referred to as "diabetic mASCs," whereas cells harvested from Kimba mice are referred to as "healthy mASCs." All mice were maintained on a C57BL/6 background. The Kimba hVEGF transgene is expressed only in retinal photoreceptors from postnatal day 9 to postnatal day 28 [16]; thus, we assumed that mASCs procured from fat pads harvested from Kimba mice at 9 weeks are unlikely to be affected by the short-term hVEGF pulse in the retina that terminated 5 weeks prior to mASC harvest. mASCs were maintained at 37°C and 5% CO₂ and cultured and passaged as previously reported [7]. Briefly, growth medium consisted of 10% fetal bovine serum (Hyclone, Logan, UT, http://www.hyclone.com) and 1% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA, http://www.thermofisher.com) in Dulbecco's modified Eagle's medium F-12 with added glutamate and sodium bicarbonate (Life Technologies, Rockville, MD, http://www.lifetech.com). Cells were passaged at roughly 80% confluence, and the culture medium was changed every other day.

Vital Labeling mASCs for Intravitreal Injection

All animal studies were approved by the University of Virginia's Animal Care and Use Committee. The presence of the Akita genotype was detected by RT-PCR [15], and hyperglycemia was confirmed by taking blood glucose measurements at 5 weeks of age using a OneTouch UltraMini blood glucose meter (Life-Scan, Milpitas, CA, http://www.lifescan.com). At passage 4 (P4), mASCs were fluorescently labeled with Vybrant Dil cell-labeling solution (Life Technologies) as per the manufacturer's instructions and resuspended in PBS at the appropriate concentration determined by hemocytometer. Each cell injection consisted of 10,000 mASCs suspended in 1.5 μ l of PBS. Control vehicle injections consisted of 1.5 μl of PBS. The cells were resuspended by pipette immediately before injection to minimize cell clumping. Five-week-old, male Akimba mice were anesthetized with ketamine/xylazine injected intraperitoneally, and proparacaine was applied topically to the eyes just prior to injection. mASCs were injected through the pars plana into the vitreous using a 33-gauge Hamilton syringe. A total of 12 Akimba mice were injected in this manner, with mASCs injected into one eye (with six randomized mice receiving healthy mASCs and six mice receiving diabetic mASCs) and PBS control injected into the other eye.

Retinal Whole-Mounting and Immunostaining

Treated mice were maintained in a controlled vivarium for 4 weeks postinjection before harvesting retinae. At the harvest time point, mice were euthanized with carbon dioxide, followed immediately by cardiac perfusion-fixation by cutting the right atrium and injecting the left ventricle with 10 ml of 4% paraformaldehyde (PFA). Intact eyes were then removed and fixed by submersion in 4% PFA for 10 minutes. After rinsing the eyes with PBS, retinae were isolated and whole-mounted on gel-coated slides. The retinae were then permeabilized with 1 mg/ml digitonin (MP Biomedical, Solon, OH, http://www.mpbio.com) for 1 hour, stained with 1:100 IB4 lectin Alexa Fluor 647 (Life Technologies) to visualize blood vessels, and counterstained with 1:500 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) to visualize cell nuclei.

Imaging and Image Analysis

Image stacks at \times 10, \times 20, and \times 60 were taken of retinal whole mounts on a Nikon (Tokyo, Japan, http://www.nikon.com) Ti Eclipse confocal laser scanning microscope. The imes10 stacks were flattened and tiled into whole-retina montages using ImageJ. Vessel length density was measured by a blinded observer in six random representative fields of view (FOV) (×10 magnification) in each retina by manually tracing and measuring the length of all vessels within the FOV using ImageJ. This number was normalized to the total tissue area in each FOV by dividing the total vessel length by the total tissue area within the FOV. In the same imes10 images, the number of vessel branch points were counted and similarly normalized by the total tissue area within the FOV. The normalized vascular length density (vessel length/tissue area) and the normalized number of branch points (branch points/ tissue area) were averaged across all six FOVs so that each retina was described by an average vascular length density and an average number of branch points (n = 6 FOVs per retina). For both the vascular length density and the number of branch points, we calculated the percent change between the mASC-injected retina and contralateral PBS-injected retina in the same mouse. Dillabeled mASCs were counted in three representative imes20 image stacks per retina by a blinded observer. Each FOV was taken at the same distance from the optic nerve.

Monte Carlo Simulation

A Monte Carlo simulation was created in MATLAB (MathWorks, Natick, MA, http://www.mathworks.com) to determine the distribution of randomly placed mASCs that would be expected to be associated with the retinal vasculature by chance. The simulation used binary images of fluorescent retinal vasculature micrographs (\times 20 magnification) to generate a matrix of coordinates for the retinal vasculature. For each micrograph, a random matrix of mASC coordinates was generated. Identical retinal vasculature coordinates and mASC coordinates were counted to determine the probability of mASCs randomly contacting retinal vasculature after random distribution. The simulation was looped 1,000 times for more accurate probability calculations. Using Delaunay triangulation, the simulation also created a histogram to visualize the probability distribution of the distance of randomly simulated mASCs from retinal vasculature. These simulated predicted distributions and associations with the retinal vasculature were then compared with the actual observed association of mASCs with the retinal vasculature. This allowed us to determine whether

mASCs were associated with the retinal vasculature at a rate greater than that expected by chance.

Acquisition of mASC-Conditioned Medium and Angiogenesis Secretome Analysis

Healthy, wild-type mASCs and diabetic mASCs were raised to passage 4 in 10% FBS, as specified above, and they were plated at densities to ensure approximately equivalent cell numbers to collect cell conditioned media from equal sized passage 4 populations. The medium was removed from culture plates, and plates were then washed with PBS twice. Defined medium lacking serum was then added to mASC culture plates for 24 hours as cells incubated at 37°C. ASC-conditioned medium samples were collected for analysis from four age-matched populations each of healthy Kimba and diabetic Akimba mASCs, taken from mice of equal age. These fresh media samples were run on a Mouse Proteome Profiler Angiogenesis Array (catalog no. ARY015; R&D Systems, Minneapolis, MN, http://www.rndsystems.com), which tested for 53 factors. X-ray film captured chemiluminescence from each dot, which was proportional to the amount of factor bound. Relative expression levels were obtained from densitometry analysis (ImageJ) of x-ray film spots. Raw intensity values were normalized to blot area, cell number, and positive control value, and then the background value was subtracted from the normalized intensity.

mASC-Conditioned Medium Injections in Akimba Mice

Male, 5-week-old Akimba mice were anesthetized with ketamine/ xylazine injected intraperitoneally and proparacaine applied topically to the eyes just prior to injection. Cell conditioned medium (CM) was obtained from healthy and diabetic mASCs according to the procedure described in Materials and Methods. Cell CM was concentrated so that the concentration of the cell secretome was equal to that produced by the number of ASCs injected in other experiments (10,000 ASCs per 1.5 μ l) using an Amicon Ultra-15 Centrifugal Filter (EMD-Millipore, Darmstadt, Germany, http://www. emdmillipore.com). Then, 1.5 µl of medium conditioned by diabetic mASCs was injected into the left eye, whereas CM from healthy mASCs was injected into the right. Injections went through the pars plana, into the vitreous using a 33-gauge Hamilton syringe (n = 6Akimba mice). Four weeks postinjection, mice were anesthetized and euthanized as described in Materials and Methods, and retaine was removed, immunostained, and imaged according to the procedures described in Materials and Methods.

Cell Proliferation and Apoptosis Assays

mASCs from three 9-week-old healthy Kimba and three 9-weekold diabetic Akimba animals were plated on glass coverslips in 12-well dishes at a density of 4,000 cells per cm² and allowed to adhere for 24 hours. Three coverslips were cultured per animal. To measure cell proliferation in each population of mASCs, mASCs were incubated with 5-ethynyl-2'-deoxyuridine (EdU) for 12 hours and then stained following the manufacturer's instructions. To measure apoptosis, the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method was used. Fluorescence intensity from both EdU and TUNEL assays was measured for each coverslip using confocal microscopy, taking image stacks with 3- μ m spacing and measuring total fluorescence intensity for each coverslip in ImageJ.

Statistical Analyses

The vascular length density and branch point analysis in PBSinjected versus ASC-injected retinae were statistically analyzed using a Mann-Whitney rank sum test. The statistical difference between the average ratios of ASCs residing in perivascular locations relative to the total number of ASCs in the retina in healthy versus diabetic ASC-injected retinae was assessed using Wilcoxon signed rank test. The in vitro cell proliferation and apoptosis mASC experiments were statistically analyzed using Student's *t* test. The in vitro angiocrine secretome data were statistically assessed by comparing healthy ASC secretome levels to diabetic ASC secretome levels for each factor using a Mann-Whitney rank sum test. Statistical tests were performed in SigmaStat (Systat Software Inc., San Jose, CA, http://www.systat.com), with statistical significance asserted as $p \leq .05$.

RESULTS

Incorporation of Injected mASCs Into the Retina

Ten thousand Dil-labeled, P4 mASCs derived from either healthy or diabetic mice were injected in 1.5 μ l of PBS into the vitreous of 5-week-old male Akimba mice, and 1.5 μ l of PBS was injected into the contralateral eye as vehicle control. Four weeks postinjection of cells, retinae were harvested demonstrating Dil-positive cells that had engrafted into the retina and had DAPI+ nuclei, some of which were associated with the retinal vasculature residing in perivascular locations (Fig. 1). The number of mASCs that incorporated into the retina per 1,250-mm² FOV did not significantly differ between retinae injected with healthy and diabetic mASCs (6.77 healthy mASCs/FOV, 6.73 diabetic mASCs/FOV).

Protection Against Vascular Dropout

The Akimba retina demonstrates several hallmarks of proliferative diabetic retinopathy seen in humans, including retinal thinning, retinal edema, aberrant neovascularization, retinal capillary dropout, and nonperfusion [15]. When healthy mASCs were injected, the average vascular density of the superficial vascular plexus in the Akimba retinae (n = 6) was significantly increased by 10.3% compared the contralateral PBS-injected retinae (p = .015) (Fig. 2A). However, when diabetic mASCs were injected, the average vascular density was decreased by 5.7% compared with the contralateral PBS-injected retinae, but this decrease was not statistically significant (n = 6, p = .82) (Fig. 2C). Similarly, there were 20.2% more branch points in the retinal vasculature in eyes injected with healthy mASCs relative to the number of branch points in the contralateral PBS-injected controls (p = .029) (Fig. 2B). Finally, there were 13.8% fewer branch points in the retinal vasculature of eyes that were injected with diabetic mASCs relative to the number of branch points in the contralateral PBS-injected controls (p = .71) (Fig. 2D). No differences were seen in the development of subretinal neovascularization in either healthy or diabetic ASC-injected eyes as compared with PBS-injected controls.

Incorporation of Injected mASCs Into Perivascular Locations

We and others have previously shown that when ASCs are injected into different tissues in vivo [17–20], including the retina [7], they exhibit pericyte-like behaviors and archetypical pericyte-



Figure 1. Injected ASCs incorporate into the retina, and some reside in perivascular locations along the vasculature (green; IB4 lectin staining). **(A, B):** Healthy ASC-treated **(A)** and diabetic **(B)** ASC-treated retinae had similar numbers of engrafted DiI+ mouse ASCs (red). DAPI staining (gray) confirmed intact nuclei of the DiI+ ASCs 1 month after treatment (yellow arrows). Cells are found in both the interstitial space and along IB4 lectin-stained vessels (green). The scales in **(A)** and **(B)** are the same. Scale bar = 50 μ m. Abbreviations: ASC, adipose-derived stem cell; DAPI, 4',6-diamidino-2-phenylindole.

like morphology. In these previously published studies, injected ASCs have been observed to reside in perivascular locations, enwrap capillaries, and modulate vascular length density in settings of injury and disease. In our study, approximately 60% of injected healthy mASCs were observed in perivascular locations (Fig. 3), whereas a significantly smaller percentage of diabetic mASCs were observed in perivascular locations (n = 16 healthy, n = 19 diabetic, p < .05). To determine whether the injected mASCs preferentially resided in perivascular positions or were localized there by chance, we carried out a Monte Carlo simulation on each $\times 20$ FOV. This stochastic simulation provided an estimate of the number of cells for each given field that one would expect to find in contact with a vessel by chance alone based on the observed vascular density and a random distribution of mASCs (Fig. 4). A larger number of data points were found on the upper left side of the diagonal for both healthy and diabetic mASCs, indicating that the number of mASCs found in contact with the retinal vasculature was greater than what was expected by chance alone for both healthy and diabetic mASCs. However, a slightly greater number of healthy cell-treated points were found above the line (62%) than diabetic cell-treated points (57%), suggesting a marginally higher preference of healthy cells for perivascular incorporation.

Healthy and Diabetic mASC Viability and Apoptosis

Relative rates of proliferation and apoptosis between healthy and diabetic mASCs were quantified in vitro using P4 cultured mASCs. Proliferation frequencies were measured using an EdU incorporation assay (Life Technologies). Diabetic mASC proliferation activity was 77 \pm 5% that of healthy mASCs (Fig. 5A). Diabetic mASC apoptosis was 121 \pm 3% that of healthy mASCs, as determined by a TUNEL assay (Fig. 5B) (n = 3 healthy and 3 diabetic animals with 3 cell plates per animal; \pm SEM; p < .05).

Healthy and Diabetic mASC Cellular Bioenergetics

An analysis of the bioenergetic profiles of healthy mASCs and diabetic mASCs was then performed using the Seahorse instrument to determine whether differences in cellular metabolism may account for their differing treatment efficacy and function in vivo.



Figure 2. Treatment with healthy versus diabetic mouse ASCs have differential effects on the Akimba mouse retinal microvasculature. The percent difference in vascular length density for each mouse between ASC-injected retinae and contralateral PBS-injected retinae showed a significant (p = .015; Mann-Whitney rank sum test) increase in healthy ASC-injected retinae (**[A]**, 10.28% increase) yet no significant change in diabetic ASC-injected retinae (**[C]**, 5.71% decrease) relative to contralateral PBS-injected retinae (p = .82; Mann-Whitney rank sum test). Healthy ASC-injected retinae (p = .82; Mann-Whitney rank sum test). Healthy ASC-injected retinae (p = .82; Mann-Whitney rank sum test). Healthy ASC-injected retinae also showed a statistically significant (p = .029; Mann-Whitney rank sum test) increase in vessel branch points (**[B]**, 20.17% increase) relative to PBS-injected retinae showed a ASC-injected retinae (**[D]**, 13.77% decrease) (p = .71; Mann-Whitney Rank Sum test). The dashed line indicates mean percent change between ASC-injected and PBS-injected contralateral control eye. Abbreviations: ASC, adipose-derived stem cell; PBS, phosphate-buffered saline; VLD, vascular length density.



Figure 3. Injected healthy mouse adipose-derived stem cells (mASCs) that have engrafted into the retina assume perivascular positions more often than diabetic mASCs. Engrafted mASCs occupying perivascular and nonperivascular positions were counted in representative $\times 20$ confocal image stacks of whole mount Akimba retina. The average ratio of perivascular cells to total engrafted cells in each field is shown for both healthy and diabetic mASCs: n = 16 fields for healthy and n = 19 fields for diabetic. *, p < .05 (Wilcoxon signed rank test).

Specifically, mitochondrial bioenergetics in whole cells was evaluated by measuring oxygen consumption over time following the sequential addition of the ATP synthase inhibitor oligomycin, the itochondrial uncoupler FCCP, and the complex I and III inhibitors rotenone and antimycin A [21]. These data revealed that diabetic and nondiabetic mASCs had comparable rates of basal cellular respiration, ATP-dependent respiration, spare respiratory capacity, uncoupled respiration, nonmitochondrial respiration, and extracellular acidification (supplemental online Fig. 1; more detail is given in the supplemental online data).

Angiogenesis Factor Secretome of Healthy and Diabetic mASCs

Levels of angiogenic factors secreted by ASCs were measured by analyzing conditioned media samples that were collected from P4 healthy and diabetic mASCs (n = 4 independent samples per group, each group was analyzed in duplicate, and each sample was obtained from ASCs sourced from a different mouse) using high-throughput enzyme-linked immunosorbent assay. Each sample was run on a separate array under identical conditions, which enabled us to calculate a relative abundance of each angiogenesis factor by comparing arrays after normalizing to the number of cells that produced the sample. Four angiogenic factors were secreted at significantly higher levels by healthy mASCs than diabetic mASCs, namely insulin-like growth factor binding protein-3 (IGFBP-3), monocyte chemoattractant protein-1 (MCP-1), osteopontin, and stromal cell-derived factor (SDF-1) (n = 4, p < .01) (Fig. 6).

To determine whether the angiogenesis factor secretomes of the healthy and diabetic mASCs were capable of affecting the retinal vasculature in the Akimba disease model, healthy mASCconditioned medium was injected into one eye, and diabetic mASC CM was injected into the contralateral eye in 5-week-old Akimba mice (n = 6 mice; all mASCs cultured in high glucose, under standard culture conditions). Four weeks later, the retinae were harvested, and lectin labeling of blood vessels revealed that a single injection of CM did not protect retinal vessels against dropout in the Akimba model to the same extent that a single injection of healthy or diabetic mASCs did, regardless of ASC origin (supplemental online Fig. 2).



Figure 4. Stochastic analysis of mouse adipose-derived stem cell (mASC) spatial distribution in the retina reveals a preference of mASCs to incorporate near retinal vessels. Each analyzed \times 20 confocal image of the retinal whole mount is represented as a data point, with the model-predicted percentage of perivascular cells for each image on the *x*-axis, and the percentage actually observed on the *y*-axis. At the dashed line (*y* = *x*), the observed percentage of perivascular cells is exactly what would be expected by chance. Above this line, a greater number of perivascular cells were observed than expected by chance, with overall both diabetic and healthy mASCs observed in greater frequency on vessels than would be predicted from a random distribution.

DISCUSSION

ASC therapy holds promise for many debilitating and increasingly common vascular disorders because of the stabilizing effects of ASCs on the microvasculature. This has been attributed to the ability of ASCs and other stem cells to release vasoprotective cytokines and to differentiate into vascular and vascular support cells, such as pericytes [20, 22, 23]. Furthermore, isolation of ASCs has several advantages over other cell types because of the relative abundance of adipose tissue and relatively painless extraction procedure [24]. The fact that ASCs can be rapidly isolated at high enough quantities for treatment without culture and expansion holds great implications for autologous treatment options. Thus, it is important to assess whether or to what extent ASCs isolated from a diabetic source have compromised treatment efficacy.

In this study, we have demonstrated that intraocularly injected mASCs harvested from diabetic mice have an impaired ability to protect retinal vasculature against progressive pathological vessel dropout as compared with ASCs harvested from nondiabetic mice. In addition to highlighting the effect of diabetes on mASCs treatment efficacy in vivo, this experiment corroborated our group's previous findings that healthy ASCs can both protect and accelerate regeneration of damaged retinal microvasculature [7]. In an effort to explain the observed decreased treatment efficacy when using ASCs from diabetic sources, we carried out an analysis of mASC incorporation in vivo and an in vitro functional analysis. We found that mASCs from diabetic sources were less likely to reside in perivascular locations along



Figure 5. Healthy mouse adipose-derived stem cells (mASCs) exhibit higher rates of proliferation and lower rates of apoptosis than diabetic mASCs. **(A)**: Relative number of mASCs in the S phase determined by 5-ethynyl-2'-deoxyuridine incorporation assay. **(B)**: mASCs undergoing apoptosis determined by terminal deoxynucleotidyl transferase dUTP nick end labeling assay. mASCs from three healthy and three diabetic animals were used for each condition, with three cell plates per animal. The values were normalized to the healthy value in each case. *, p < .001 (Student's *t* test).

vessels in the retina compared with mASCs derived from healthy sources. When cultured in vitro, mASCs from diabetic donors underwent decreased proliferation and increased apoptosis, in accordance with other studies that have compared diabetic to healthy ASCs [4, 25]. However, a statistically similar number of diabetic ASCs and healthy ASCs engrafted in the retina 1 month after injection, underscoring the fact that cells growing in wellcontrolled culture conditions can behave guite differently than cells injected in vivo, especially if pathological conditions are present [26]. Hence, we cannot rule out the possibility that the diabetic and nondiabetic ASCs that engraft into the retina may have similar proliferation rates, and it is also possible that the engraftment process either normalizes proliferation rates between diabetic and nondiabetic ASCs (i.e., by slowing down nondiabetic ASC proliferation or speeding up diabetic ASC proliferation) or selects for a subpopulation of ASCs with a certain proliferation capacity. Analysis of mASC bioenergetics revealed no differences between healthy and diabetic mASCs, but analysis of their secretome profiles differed with respect to four factors that have been implicated in vascular stability, as discussed in more detail below.

The mechanisms by which mASCs protect the retinal vasculature and the underlying failure of diabetic mASCs to perform this function, as well as nondiabetic mASCs, remain unclear. Pericytes belong to the vascular smooth muscle cell lineage [27] and are known to communicate with endothelial cells through paracrine, juxtacrine, and direct cell-cell contact. ASCs have been found to have many similarities with pericytes, such as expressing pericyte surface markers, taking up a perivascular location, and stabilizing endothelial networks [17-19, 28]. An absence of pericytes, such as in the pathogenesis of diabetic retinopathy, leads to loss of endothelial cells, dysfunction of endothelial cell junctions, and abnormal vessel morphology [29]. Mesenchymal stem cells (MSCs) are thought to be closely connected with perivascular cells [30, 31], and ASCs have been shown to originate from perivascular MSCs, which reside in great numbers in adipose tissue [29]. This would suggest that ASCs might exert their vascular-stabilizing effects in the retina by effectively substituting for decreased native pericyte activity either through replacing their paracrine functions or directly taking up retinal perivascular positions, as we have observed in this and our prior study.



Figure 6. Diabetic mouse ASCs (mASCs) secrete lower levels of four factors that have been shown to promote vascular stability. Relative expression levels of secreted angiogenesis factors by healthy versus diabetic mASCs were obtained using high-throughput enzyme-linked immunosorbent assay arrays. mASCs were isolated from "healthy" Kimba (black bars) and "diabetic" Akimba (gray bars) mice and maintained to passage 4. Samples consisted of conditioned media obtained by incubating cells immediately after passage in fresh, serum-free media, and collecting media after 24 hours. Relative expression levels were obtained from densitometry analysis (ImageJ) of x-ray film spots. Raw intensity values were normalized to blot area, cell number, and positive control value, and then the background value was subtracted from the normalized intensity. n = 4 mASC populations from 4 mice per group. *, p < .01 (Mann-Whitney rank sum test). The values are presented as means \pm SEM. Abbreviations: ASC, adipose-derived stem cell; FGF, fibroblast growth factor; IGFBP-3, insulin-like growth factor binding protein-3; MCP-1, monocyte chemoattractant protein-1; SDF-1, stromal cell-derived factor.

Several studies have found that the regenerative effects of MSCs are at least in part due to the release of paracrine factors [32-34], which act on the microvasculature by facilitating endothelial progenitor cell homing and restructuring vascular networks [31]. A comparison of diabetic and healthy embryonic stem cell function using a hind limb ischemia model also determined that the loss of function and treatment efficacy was due to lower angiogenic factor secretion by diabetic ESCs [35]. Consistent with these prior studies, we find that mASCs from diabetic mice secrete a milieu of growth factors and chemokines in vitro that differ markedly from that secreted by ASCs harvested from nondiabetic mice. In particular, mASCs from diabetic sources secreted lower levels of known retinal vasoprotective and neuroprotective factors including IGFBP-3, MCP-1, osteopontin, and SDF-1 [36-39]. Each of these factors has been associated with increased stability and preservation of the vasculature in retina and other tissues. Notably, secretome differences persist whether the mASCs are raised in high or physiologic glucose, except for osteopontin, which under physiologic glucose conditions has similar levels in diabetic and nondiabetic mASCs (data not shown). For all of the studies we report here, we cultured mASCs in conventional (high) glucose conditions because mASCs were subsequently injected into the chronic hyperglycemic environment of the Akimba eye and remained there for the duration of the month prior to harvest.

Although we observed vasoprotective effects of mASCs on the superficial vasculature, importantly we do not find increases in aberrant neovascularization, which characteristically develops in the outer nuclear layer in the Akimba mouse [15]. The primary vasoprotective effect of mASCs on the superficial retinal vasculature may be secondary to decreased inflammation or a rebalancing between proangiogenic and antiangiogenic factors in the Akimba retina. Both mechanisms have been demonstrated to control vascular stability during development and vascular network formation and appear to be characteristic of ASC function [7, 17–20]. The current results suggest that diabetic mASCs are less effective at restoring stability to the highly unstable Akimba retinal vasculature, although whether this is due to reduced sensing of versus response to the microenvironment associated with this progressive retinopathy remains unclear.

Surprisingly, we were unable to recapitulate the vasoprotective effects of injected healthy mASCs through use of concentrated medium conditioned by these cells. This runs counter to previous studies that found ASC conditioned medium was neuroprotective against light-induced retinal toxicity [40]. The reason for this discrepancy remains unclear but could be explained by the fact that in the work of Tsuruma et al. [40], the conditioned medium injection was performed prior to retinal insult, retinal neural cells rather than the vasculature were assessed, and an earlier time point (5 days) than what was evaluated in our study (1 month) was assessed. It is possible that the levels of growth factors and cytokines that can be achieved with injection of concentrated conditioned media are subthreshold compared with that achieved with direct ASC injection or that ASCs are responding to the microenvironment of the Akimba retina in a manner markedly different than they do in culture. It is also possible that direct injection of ASCs has more anti-inflammatory effects than injection of conditioned medium does on the retina, which would be expected to improve retinal vascular protection [41]. Regardless, our present findings would argue that direct ASC injection into the eye, including autologous diabetic ASCs, may have higher therapeutic potential than injection of derivative cell conditioned products for the treatment of diabetic retinopathy.

It is interesting to note that although diabetic mASCs showed decreased secretion of these vasoprotective factors, diminished ability to home to and reside in perivascular locations, and an inability to protect the retinal vasculature from dropout, impairments were not universally seen in all aspects of cell function. Specifically, metabolic capacity and mitochondrial function were identical in healthy and diabetic cells, under both oxidative respiration and glycolysis conditions, suggesting that diabetesassociated hyperglycemia had no lasting effect on these particular cellular functions (supplemental online Fig. 1). Although others have reported significant hyperglycemia-induced changes in metabolism in other cell types such as bovine retinal pericytes, these studies were performed using high-glucose media [42]. The fact that we saw no metabolic differences after culturing cells to passage 4 indicates that these differences were likely transient, in contrast to changes in the secretome, which was persistent.

Recent evidence implicates epigenetic modification as a driving force in diabetic microvascular complications, which are thought to lead to dysregulation of oxidant and proinflammatory factors and promote vascular inflammation [43–45]. MSCs are well-known for their anti-inflammatory role [46] and have been hypothesized to transition between multiple states in response to local cues during infection and wound healing [47]. The mechanism of epigenetic modifications in MSCs or ASCs in response to hyperglycemia is as of yet unknown, but it is possible that these modifications cause similar proinflammatory changes in ASCs and are responsible for the observed differences in cell survival and cytokine secretion.

The observed therapeutic differences in mASCs isolated from healthy and diabetic sources, as well as their cell conditioned media, have implications for the use and study of stem cells beyond the model disease in this study. Cell conditioned medium, at least for our model system, appears to lack the full complement of regenerative factors needed to protect the retinal vasculature. Based on the fact that diabetic mASCs are impaired in their regenerative ability to the point that their use did not elicit a predictable and positive response in vivo, an autologous approach to ASC therapy in diabetic patients requires careful consideration of the need to correct for these differences. The apparent dysfunction of diabetic perivascular ASCs in the fat may be reflective of the known dysfunction of retinal pericytes in the diabetic retina [48]. Furthermore, the greater levels of vasoprotective factor secretion in healthy mASCs and the concomitant increase in retinal microvascular regeneration supports the notion that allogeneic approaches may ultimately be more effective for treating diabetic retinopathy.

CONCLUSION

We have shown that ASCs taken from diabetic sources have an impaired ability to stabilize the microvasculature in diabetic retinopathy and that use of autologously derived ASCs from diabetic patients may not be as effective in protecting against vascular dropout in the retina. Furthermore, hyperglycemia causes distinct changes in ASC function, but only to certain aspects related to regulation of inflammation and vasoprotection. To improve ASC therapy for diabetic retinopathy, as well as other diabetic complications, it is critical to better understand their mechanism of action in vivo. Future work may focus on describing the means by which ASCs migrate toward and incorporate into the retina and their activity in vivo, as well as the mode of diabetes-induced changes in ASC function.

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AUTHOR CONTRIBUTIONS

S.M.C. and T.A.M.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, and final review of manuscript; M.R.K.-G.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final review of manuscript; H.C. R.: data analysis and interpretation, final review of manuscript; K. L.H.: conception and design, provision of study material, collection and/or assembly of data, data analysis and interpretation; A.C.B.: review of manuscript; B.K.D.: conception and design, collection and/or assembly of data, final review of manuscript; A.M. G.: administrative support, collection and/or assembly of data, final review of manuscript; D.N.T.: collection and/or assembly of data, data analysis and interpretation, final review of manuscript; I.M.H., S.M.P., and P.A.Y.: conception and design, financial support, data analysis and interpretation, manuscript writing, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

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