



Published in final edited form as:

*Stem Cells*. 2009 July ; 27(7): 1686–1696. doi:10.1002/stem.87.

## Bone Marrow Mononuclear Cells Have Neurovascular Tropism and Improve Diabetic Neuropathy

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### Abstract

Bone marrow-derived mononuclear cells (BMNCs) have been shown to effectively treat ischemic cardiovascular diseases. Because diabetic neuropathy (DN) is causally associated with impaired angiogenesis and deficiency of angiogenic and neurotrophic factors in the nerves, we investigated whether DN can be ameliorated by local injection of BMNCs. Severe peripheral neuropathy, characterized by a significant decrease in the motor and sensory nerve conduction velocities (NCVs), developed 12 weeks after the induction of diabetes with streptozotocin in rats. The injection of BMNCs restored motor and sensory NCVs to normal levels and significantly improved vascular density and blood flow in diabetic nerves over 4 weeks. Fluorescent microscopic observation revealed that DiI-labeled BMNCs preferentially engrafted in sciatic nerves. Whole-mount fluorescent imaging and confocal microscopic evaluation demonstrated that many of the BMNCs localized following the course of the vasa nervorum in close proximity to blood vessels without incorporation into vasa nervorum as endothelial cells at a detectable level. Real-time reverse transcription-polymerase chain reaction analysis showed that the levels of angiogenic and neurotrophic factors were significantly increased in the nerves by BMNC injection. Local transplantation of BMNCs improved experimental DN by augmenting angiogenesis and increasing angiogenic and neurotrophic factors in peripheral nerves. These findings suggest that BMNC transplantation may represent a novel therapeutic option for treating DN.

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**Disclosure of Potential Conflicts of Interest:** The authors indicate no potential conflicts of interest.

Author contributions: H.K.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; J.S.P.: conception and design, collection and/or assembly of data, data analysis and interpretation; Y.J.C.: collection and/or assembly of data, contributing experimentation, administrative support; M.O.K.: manuscript writing, final approval of manuscript; Y.H.H.: collection and/or assembly of data; S.W.K.: collection and/or assembly of data; J.W.H.: collection and/or assembly of data; J.Y.L.: collection and/or assembly of data, contributing experimentation, administrative support; S.K.: contributing experimentation; M.A.H.: contributing experimentation; M.I.: contributing experimentation; Y.S.Y.: conception and design, financial support, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript.

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## Keywords

Bone marrow mononuclear cells; Diabetes; Diabetic neuropathy; Angiogenesis; Angiogenic factors; Neurotrophic factors

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## Introduction

Diabetes is a rapidly growing global health problem [1] and peripheral neuropathy is the most common complication of diabetes, affecting up to 60% of long-standing diabetic patients [2]. Diabetic neuropathy (DN) is characterized by loss of sensation in the feet, leading to a failure to perceive traumatic injuries, which can ultimately result in skin ulceration and even limb amputation [3,4]. Several pathogenic mechanisms have been proposed for DN: nonenzymatic glycation of proteins important for neural function [5], alteration in neural polyol metabolism [6,7], decrease in neurotrophic factors [3], production of reactive oxygen species [8–10], degeneration of dorsal root ganglion associated with mitochondrial damage [11], and microvascular disease with impaired blood circulation in diabetic nerves [7,12,13]; however, there is currently no effective therapy for DN.

Previously, our group has shown that experimental DN is characterized by reduced blood flow in the nerves and destruction of vasa nervorum. We also found that administration of angiogenic factors such as vascular endothelial growth factor (VEGF)-A, VEGF-C, sonic Hedgehog (SHh), and statin restored microcirculation in the affected nerves accompanied by functional improvement of the nerves [13–15]. On the other hand, the lack of neurotrophic factors has been regarded as an important pathogenic mechanism of DN [2,16]. Administration of neurotrophic factors, such as nerve growth factor (NGF) [17], insulin-like growth factor (IGF)-1 and IGF-2 [18,19], ciliary neurotrophic factor [20], or glial cell line–derived neurotrophic factor [21], is known to ameliorate DN in animal models. Interestingly, growing evidence demonstrates that many classical angiogenic factors have neurotrophic activities and many neurotrophic factors have angiogenic effects [22]. VEGF [23], IGF-1 [18,19], NGF [24–26], brain-derived neurotrophic factor [27,28], endothelial nitric oxide synthase (NOS)-3 [29], and fibroblast growth factor (FGF)-2 [30,31] are all examples of factors with these dual effects. These findings suggest that a therapeutic modality that augments angiogenesis and provides angiogenic and neurotrophic factors can contribute to treatment of DN.

Therefore, cell therapy using bone marrow (BM)-derived mononuclear cells (BMNCs) can be one of the best treatment options, because transplantation of BMNCs has been shown to augment neovascularization and increase a broad range of angiogenic and/or neurotrophic factors, including FGF-2, VEGF, and angiopoietin-1, in the tissue [32,33]. In animal models, transplantation of BMNCs into ischemic limbs [33] and myocardium [32] increased neovascularization and collateral vessel formation associated with increased local concentrations of angiogenic factors. This effect of BMNCs has also been documented in human patients with limb ischemia in randomized controlled trials [34]. The therapeutic effects of BMNCs are not surprising, given that BMNCs include endothelial progenitor cells (EPCs) and marrow stromal cells (MSCs), both of which are known to induce neovascularization in ischemic tissues, leading to enhanced recovery from ischemic insults [35–38]. EPCs increase neovascularization by transdifferentiating into endothelial cells (ECs), secreting angiogenic factors, and inducing angiogenic factor expression from host tissues as well [39,40]. MSCs express a broad spectrum of angiogenic factors, and local injection of MSCs increased tissue levels of angiogenic factors such as VEGF and FGF-2 [37,38]. However, EPCs and MSCs need to be grown in culture to be used for treatment, which takes time and presents technical difficulties. In contrast, BMNCs are easily isolated and do not have to be expanded by ex vivo culture. This ease of isolation makes BMNCs a more attractive source of cells for therapeutic

neovascularization. In this regard, we investigated whether local transplantation of BMNCs can attenuate or reverse DN in a rat model of DN.

In this study, we found, for the first time, that i.m. transplanted BMNCs preferentially localized to diabetic nerves, especially around vasa nervorum, and increased expression of various angiogenic and neurotrophic factors in the nerves. The vascularity of the diabetic nerves was improved and the nerve conduction velocities were restored to near normal.

## Materials and Methods

### Induction of Diabetes

All animal protocols were approved by the Institutional Animal Care and Use Committees at Caritas St. Elizabeth's Medical Center and Emory University School of Medicine. We induced diabetes in 8-week-old male Fischer 344 rats by i.p. injection of streptozotocin (75 mg/kg in 0.9% sterile saline). Serum glucose levels were measured 2 weeks later, and all animals with glucose levels <300 mg/dl were excluded from the study. These diabetic (DM) rats were randomly assigned to BMNC or saline injection groups 12 weeks after the induction of diabetes (supporting information Fig. 1). Age- and sex- matched rats were used as nondiabetic (NDM) controls and received the same treatment.

### Isolation and Transplantation of BMNCs

The femur, tibia, and iliac bones of 8-week-old male Fischer 344 rats were excised and all connective tissues attached to the bones were removed. The bones were crushed using a mortar and pestle in phosphate-buffered saline (PBS) supplemented with 1 mM EDTA, and bone fragments and connective tissue were removed with 70- $\mu$ m sieves to obtain BM cell suspensions. Mononuclear cells were isolated by density centrifugation over Histopaque-1083 (Sigma-Aldrich, St. Louis, <http://www.sigmaaldrich.com>) according to the manufacturer's instructions. We labeled BMNCs with DiI (Molecular Probes Inc., Eugene, OR, <http://probes.invitrogen.com>) according to the manufacturer's instructions and, after baseline nerve conduction studies, injected five million BMNCs in 500  $\mu$ l saline or control saline into the muscles along the course of the sciatic nerve at five sites with 0.7–0.9 cm distance between each injection using a 30-gauge needle.

### Measurements of Nerve Conduction Velocity

Nerve conduction velocities (NCVs) were measured in hindlimbs bilaterally using standard orthodromic surface recording techniques and a Teca TD-10 portable recording system (Oxford Instruments, Chicago, IL, <http://www.oxford-instruments.com>) at baseline (before treatment) and 2 and 4 weeks after treatment, as described previously [13,15]. Briefly, motor NCV was calculated by dividing the distance between stimulating electrodes by the average latency difference between the peaks of the compound muscle action potentials evoked from two sites (sciatic notch and ankle). Sensory NCV was calculated by dividing the distance between stimulating and recording electrodes by the latency of the signal from the stimulation artifact to the onset of the peak signal. For each nerve, maximal velocities were determined bilaterally.

### Laser Doppler Perfusion Imaging of Vasa Nervorum Blood Flow

Blood perfusion of sciatic nerves was measured bilaterally in each hindlimb of rats with a laser Doppler perfusion imager (LDPI) system (Moor Instruments, Wilmington, DE, <http://www.moor.co.uk>), as described previously [13,14]. Briefly, rats were anesthetized and nerves were exposed by scalpel incision and blunt dissection of the overlying muscle. The animals were placed on a heating blanket with a thermostat to keep constant temperature. The blood perfusion of nerves was measured and recorded by the LDPI system as color-coded

images. As a result of variability between individual animals, we compared the blood perfusion of the nerve of the limb injected with BMNCs with that of the contralateral limb injected with the same volume of saline.

### Measurement of Blood Flow Using Microspheres

The amount of blood flow in the nerve was determined using microspheres, with some modifications to a previously described method [41]. Briefly, the rats were anesthetized and 1 ml of 1:1 mixture of 10  $\mu\text{m}$  green- and 15  $\mu\text{m}$  red-fluorescent microspheres ( $10^6$  microspheres/ml, all from Invitrogen, Eugene, OR, <http://www.invitrogen.com>) were slowly injected over 1 minute into the abdominal aorta. The animals were euthanized and the nerves were harvested. The nerve was frozen embedded in OCT compound and cut into 100- $\mu\text{m}$  sections. The number of microspheres was determined under fluorescent microscopy and normalized to the length of the nerve.

### Fluorescent Imaging of Blood Vessels in Sciatic Nerves

Vascularity of sciatic nerves from both nondiabetic and diabetic rats was assessed by in situ fluorescent staining using the EC-specific marker BS-1 lectin conjugated to fluorescein isothiocyanate (FITC; Vector Laboratories, Burlingame, CA, <http://www.vectorlabs.com>), as described previously [13,14]. Briefly, after anesthesia and surgical laparotomy, the aorta was catheterized, the hindlimbs were perfused with FITC-conjugated BS-1 lectin (1 mg per rat), and the inferior vena cava was ligated to facilitate in situ staining of the lectin-FITC conjugate. Fifteen minutes later, the animals were sacrificed and the sciatic nerves were harvested and fixed in 4% paraformaldehyde. After fixation, samples were either whole mounted for longitudinal analysis or embedded in OCT compound for frozen sections. Samples were analyzed using a computer-assisted Nikon fluorescence microscope with digital camera (Eclipse TE200; Nikon Inc., Melville, NY, <http://www.nikoninstruments.com>). The number of vessels was counted in 10 randomly selected cross-sections under fluorescent microscopy (200 $\times$ ).

### Confocal Microscopic Analysis

Diabetic nerves were harvested 4 weeks after injection of DiI-labeled BMNCs, fixed in 4% paraformaldehyde at 4 $^{\circ}$  C for 16 hours, and incubated in 30% sucrose solution for 24 hours. Frozen sections of the nerves were obtained at a 14- $\mu\text{m}$  thickness. Blood vessels were stained with an EC marker, isolectin B4 (Vector Laboratories), as previously described [40], and nuclei were stained with 200 nM To-Pro-3 (Invitrogen). ECs were identified by positive staining for isolectin B4 and their characteristic morphology within the vascular structure. BMNCs were identified by positive DiI staining. Incorporation of BMNCs into the vasa nervorum as ECs was determined using a Zeiss LSM 510 Meta confocal laser scanning microscope (Carl Zeiss, Jena, Germany, <http://www.zeiss.com>) and LSM 510 Image software (Carl Zeiss).

### Quantitative Reverse Transcription-Polymerase Chain Reaction for Angiogenic and/or Neurotrophic Factors in the Nerves

Total RNA was extracted from sciatic nerves with Trizol (Invitrogen) according to the manufacturer's instructions. First-strand cDNA was generated using the Taqman Multiscribe Reverse Transcription (RT) Kit (Applied Biosystems, Foster City, CA <http://www.appliedbiosystems.com>). Gene expression was determined by Taqman real-time quantitative polymerase chain reaction (PCR) (7300 Sequence Detection System; Applied Biosystems) using Taqman PCR Master Mix (Applied Biosystems). Relative mRNA expression of target gene normalized to glyceraldehyde-3-phosphate dehydrogenase was calculated as previously described [40]. The primers and probes were designed using Primer Express 3.0 (Applied Biosystems) and are described in Table 1.

## Statistical Analysis

All results are presented as the mean  $\pm$  standard error of the mean. Statistical analysis was performed by Student's *t*-test for comparisons between two groups and by analysis of variance for more than two groups.  $p < .05$  was considered to be statistically significant.

## Results

### Immunophenotyping of Rat BMNCs

To characterize rat BMNCs, expression of hematopoietic cell markers was studied by flow cytometric analysis. As expected, 99% of BMNCs were positive for CD45, indicating that almost all the BMNCs were hematopoietic cells (supporting information Fig. 2A). Expression of CD3 (T lymphocyte marker), CD45R (B lymphocyte marker), and CD11b (myeloid cell marker) was observed in 3%, 79%, and 8% of BMNCs, respectively. Lineage-negative cells, which do not express any of the above lineage markers and represent the enriched hematopoietic stem and progenitor cell fraction, were about 12% of BMNCs. These data are compatible with a previous report by Alexandre et al. [42].

### Local Transplantation of BMNCs Improves NCVs in DN

To investigate the therapeutic effects of local transplantation of BMNCs on diabetic nerves, we measured the NCVs every 2 weeks for 4 weeks ( $n = 10$ , each group) after treatment. Electrophysiological study showed about a 30% decrease in both motor and sensory NCVs in diabetic rats at 12 weeks of diabetes, indicating development of a severe peripheral neuropathy (NDM-Saline versus DM-Saline: motor NCV,  $45.9 \pm 2.2$  m/s versus  $32.0 \pm 2.5$  m/s; sensory NCV,  $46.0 \pm 2.5$  m/s versus  $33.0 \pm 2.6$  m/s;  $p < .01$  for both) (Fig. 1). Local transplantation of five million BMNCs restored both motor and sensory NCVs over 4 weeks to  $41.9 \pm 3.2$  m/s and  $43.0 \pm 3.0$  m/s, respectively (both  $p < .05$  versus DM-Saline;  $p$  not significant versus NDM-Saline), whereas saline treatment did not. We further investigated the effects of diabetes and BMNC treatment on the axon and myelin structures of the nerves using toluidine blue staining and electron microscopy, because structural changes such as demyelination and axonal degeneration in the nerve can be associated with changes in the NCV. Interestingly, we did not find any visible differences regarding myelination and axonal changes among normal, diabetic control (PBS), and BMNC-treated diabetic nerves (supporting information Fig. 3, supporting information material). We also measured blood glucose levels among the treatment groups to determine the effects of local BMNC transplantation on glycemic control and to rule out the possibility that improved blood glucose levels were responsible for the improved NCVs. We found that blood glucose levels were similar between PBS- and BMNC-treated groups (PBS versus BMNC,  $391 \pm 84$  mg/dl versus  $387 \pm 55$  mg/dl), which is compatible with a previous report by Hasegawa et al. [43].

### BMNCs Increase Vascularization in Diabetic Nerves

To determine whether the transplantation of BMNCs increases the microvascular circulation of the sciatic nerve, we measured the sciatic nerve blood flow using the LDPI system 4 weeks after cell injection. Because of variability between individual animals, we compared the ratio of the blood perfusion of the nerve of the limb injected with BMNCs to that of the contralateral limb. The blood flow of the nerves was 2.1-fold  $\pm$  0.3-fold higher in the BMNC-injected rats than in the saline-injected rats (DM-BMNCs versus DM-Saline;  $p < .05$ ) (Fig. 2A, 2B). To further validate the LDPI results, we evaluated blood flow using fluorescent microspheres [41,44]. Blood flow values measured with two different sizes of microspheres (10  $\mu$ m and 15  $\mu$ m) were 1.8-fold  $\pm$  0.2-fold and 2.4-fold  $\pm$  0.4-fold higher in the BMNC-treated nerves than in the saline-treated nerves, respectively (DM-BMNCs versus DM-Saline,  $p < .05$ ) (Fig. 2C, 2D).

Next, to examine changes in the functional blood vessels at the histological level, we collected sciatic nerves 4 weeks after treatment. Before harvesting nerves, FITC-conjugated BS-1 lectin was injected i.v. to visualize functional blood vessels. Whole-mounted longitudinal (Fig. 3A) and cross-sectional (Fig. 3B) images demonstrated that the nerves from nondiabetic rats were well vascularized and had clear epineurial longitudinal networks and penetrating branches running from epineurial to endoneurial vessels. However, in diabetic rats, both the epineurial and endoneurial vessels, and especially small branches from the epineurial vessels, were markedly decreased, leaving focal areas very poorly vascularized. Transplantation of BMNCs augmented vascularity in diabetic nerves, particularly small branches of epineurial blood vessels. Quantitative analysis of vasa nervorum in the cross-sections of sciatic nerves showed that the number of blood vessels was 1.8 times higher in BMNC-transplanted nerves than in saline-injected nerves (DM-BMNCs versus DM-Saline,  $68 \pm 5.9$  versus  $37 \pm 4.4$  per cross-section;  $p < .01$ ) and was similar to the nondiabetic control nerves ( $82 \pm 6.2$  per cross-section) (Fig. 3C).

### Transplanted BMNCs Preferentially Localize to Diabetic Nerves and Vasa Nervorum

We next explored the mechanisms underlying the improvement in nerve function and vascularity following BMNC treatment. As a first step, we examined the distribution of DiI-labeled BMNCs 4 weeks after cell injection. Because we injected cells directly into the muscles of the thigh, we examined the cell fate in both muscles and sciatic nerves to investigate the cell fate in tissues. Before sacrificing rats, FITC-conjugated BS-1 lectin was injected into the abdominal aorta to visualize blood vessels in hindlimbs. Whole-mounted longitudinal images under fluorescent microscopy showed that a large number of the injected BMNCs were distributed in nerves and many of them were observed along the vasa nervorum (Fig. 4A). This close spatial relationship of homed BMNCs with vasa nervorum suggests that homed BMNCs were likely to have induced angiogenesis in the pre-existing vasa nervorum via secreting cytokines and physical interaction. In contrast, in the thigh muscles into which the BMNCs were injected, a smaller number of BMNCs was observed than in nerves (Fig. 4B). More BMNCs were observed in the fascia of muscles neighboring the sciatic nerves than within the muscle proper. To further elucidate whether differential survival or proliferation of BMNCs between nerves and muscles can cause apparently higher accumulation of BMNCs in the nerve, we examined the cell death and proliferation of the transplanted BMNCs with the terminal deoxynucleotidyl transferase dUTP nick end labeling assay and Ki67 staining, respectively. These assays revealed that there was no significant difference in the rate of apoptosis and proliferation of the transplanted BMNCs between the nerve and muscle (supporting information Fig. 4). These findings clearly support the tropism of BMNCs toward nerves over muscles in diabetes. Next, we investigated the mechanisms involved in this neurotropism of BMNCs. In our recent study, we found that two chemoattractive factors, stromal cell-derived factor (SDF)-1 $\alpha$  and monocyte chemoattractant protein (MCP)-1 are highly expressed in diabetic nerves, but not in the muscle of diabetic animals [45]. Therefore, we carried out a cell migration assay and found that diabetic BMNCs migrated to these chemotactic factors in a dose-dependent manner (supporting information Fig. 5), suggesting that these chemokines may play a role in the homing of BMNCs toward the diabetic nerve.

### Transplanted BMNCs Do Not Differentiate Into ECs in Vasa Nervorum

Because transplanted BMNCs increased the vascularity of the nerves, we examined whether BMNCs could differentiate into ECs and incorporate into vasa nervorum. Confocal microscopy revealed that many of the DiI-labeled BMNCs were engrafted in the nerves in close contact with vasa nervorum (Fig. 5). However, our examination of >2,000 transplanted DiI-labeled BMNCs in three independent experiments did not reveal colocalization of DiI-labeled cells with isolectin B4<sup>+</sup> ECs in vessels. These results suggest that BMNCs rarely, if ever, transdifferentiate into ECs in vasa nervorum. We further examined the phenotypes of the

transplanted cells. The majority of cells were positive for CD45R and CD45 at 4 weeks after transplantation (supporting information Fig. 6) as well as before transplantation (supporting information Fig. 2), suggesting that the transplanted BMNCs retain their hematopoietic phenotype in the diabetic tissues.

### BMNCs Increase the Levels of Angiogenic and Neurotrophic Genes Decreased in DN

We next determined whether transplantation of BMNCs increased expression of angiogenic and/or neurotrophic factors in the nerves. We evaluated gene expression in the nerves 2 weeks after BMNC transplantation. Real-time RT-PCR analysis showed that expression levels of angiogenic and neurotrophic genes in diabetic nerves were lower than in nondiabetic control nerves (DM-Saline over NDM-Saline: VEGF-A, 0.63-fold  $\pm$  0.08-fold; IGF-1, 0.27-fold  $\pm$  0.10-fold, both  $p < .05$ ; NOS-3, 0.02-fold  $\pm$  0.01-fold,  $p < .01$ ) and were significantly increased by BMNC transplantation (DM-BMNCs over DM-Saline: VEGF-A, 2.1-fold  $\pm$  0.2-fold; FGF-2, 2.4-fold  $\pm$  0.1-fold; NOS-3, 18.1-fold  $\pm$  5.0-fold, all  $p < .05$ ; IGF-1, 25.5-fold  $\pm$  2.0-fold,  $p < .001$ ) (Fig. 6). However, mRNA levels of SDF-1 $\alpha$ , hypoxia-inducible factor 1 $\alpha$ , and NGF- $\beta$  were not significantly different between groups.

## Discussion

In the U.S., 23.6 million people (7.8% of the population) have diabetes and about 60%–70% of diabetic patients have DN [46]. However, effective therapy for DN has yet to be developed. Here, we demonstrate that local transplantation of BMNCs improved experimental DN. We can summarize the most notable findings as follows. First, local transplantation of BMNCs restored motor and sensory NCVs of the diabetic nerves. Second, BMNC treatment induced revascularization of diabetic nerves. Third, i.m. injected BMNCs preferentially localized to diabetic nerves in close proximity to vasa nervorum, but were not colocalized with ECs and did not incorporate into vessels as ECs at a detectable level. Fourth, the expression of angiogenic and neurotrophic factors in the nerves was increased by BMNC transplantation.

In this study, we showed that the transplantation of BMNCs restored the vascularity and function of diabetic nerves, supporting the hypothesis that neural vascularity is pathophysiologically associated with the development and reversal of DN. In fact, previous publications reported improvement in experimental DN through augmentation of neural angiogenesis by administration of VEGF [13], SHh [14], and rosuvastatin [15], suggesting that the vasa nervorum can play a pathogenic role in the development and reversal of DN. This is also compatible with recent understanding that vascular defects play a crucial role in the pathogenesis of many neurological disorders (reviewed in [47]).

The increase in the number of blood vessels by BMNC treatment can be mediated by either angiogenesis (growth of new capillaries from pre-existing blood vessels) or vasculo-genesis (de novo formation of vessels from stem or progenitor cells) [48–50]. To investigate the mechanisms underlying the increase in blood vessels following BMNC transplantation, we first examined the cell fate of the injected BMNCs with confocal microscopy. From >2,000 cells counted, we were unable to find any DiI-labeled BMNCs that expressed an EC marker and were incorporated into vasa nervorum. This observation suggests that BMNCs do not transdifferentiate into nor fuse with ECs in diabetic nerves at a detectable level. Thus, it is very likely that angiogenesis is the main mechanism of BMNC-induced neovascularization in experimental DN. Further supporting this hypothesis is the increase in multiple key angiogenic factors such as VEGF, FGF-2, IGF-1, and NOS-3 in nerves by BMNC transplantation [51]. Because concerted actions of various bioactive molecules are important for inducing angiogenesis, this increase in multiple angiogenic factors is one of the main advantages of cell therapy over any single gene or protein therapy. The simultaneous neurotrophic effects of these upregulated factors [18,19,23,29–31] (reviewed in [47]) should yield additional benefits

toward the reversal of DN. Moreover, although the paracrine effects of BMNCs have been previously reported in cardiac and hindlimb ischemia models [32,33], this study is the first to show the paracrine effects of BMNCs specifically in neuropathy.

One previous study reported therapeutic effects of BMNCs in experimental DN [43]. That study showed that BMNC injection in diabetic rats improved motor NCV and increased blood flow of nerves. However, the mechanistic explanation was unclear. First, the previous study proposed arteriogenesis as the mechanism of increased blood flow in the nerve; however, direct evidence for arteriogenesis was lacking. Their suggestion was solely based on their data that blood vessel density was not changed in diabetic nerves, suggesting that angiogenesis was not enhanced by BMNC treatment. They assumed that arteriogenesis might have increased the blood flow in nerves, but they did not actually count the number of small arterioles in either the muscles or the nerves. Our data clearly show that angiogenesis was augmented in diabetic nerves by BMNC treatment. One possible explanation could be the difference in strain (Lewis versus Fisher) or duration of diabetes (8 weeks versus 16 weeks) or a combination of both. As diabetes in humans is a chronic disease and complications of diabetes develop over a long period of time, the longer duration of experimental diabetes in this study likely better reflects human DN. In fact, there are several previous studies reporting a decrease in the number of blood vessels in diabetic nerves in various strains of rats with diabetes of 12- to 16-weeks duration [13,14,52]. In our study, we provided histologic and molecular biological evidence of increased angiogenesis and potential neurotrophic effects of transplanted BMNCs. Second, the previous study did not address the localization or fate of injected BMNCs. Our histologic analysis demonstrated that i.m. injected BMNCs were preferentially engrafted into nerves, especially along the course of the vasa nervorum. This is the first study showing the neurotropism of BMNCs: BMNCs injected into muscles homed more to nerves than to muscles, and are closely positioned next to vasa nervorum within the nerves. In addition, this close localization of BMNCs to endothelial cells suggested the possibility that engrafted BMNCs might contribute to stabilizing vasa nervorum by behaving like mural cells or pericytes (Fig. 5).

Third, sensory NCV, which is a crucial parameter in DN, was not mentioned in the previous study. Loss of sensation in the feet is one of the most frequent, earliest, and characteristic symptoms of DN in humans and plays a critical role in diabetic foot, a severe secondary complication [3,4]. Finally, the dose of injected cells in the previous study was 20 times higher than in ours. The dose, one hundred million cells, is fairly large given that other studies used one million EPCs for DN in rats [52] and seven million BMNCs for hindlimb ischemia in rabbits [53], which are 10 times bigger than rats. Using minimal effective doses may avoid unnecessary side effects.

In the present study, we made some very intriguing and novel observations on the homing characteristics of injected BMNCs. The most notable finding was the neurovascular tropism exhibited by the i.m. injected BMNCs, which preferentially localized in nerve over muscle tissue and, in diabetic nerves, were positioned in close proximity to the vasa nervorum. Although neural stem cells and circulating BM-derived osteoprogenitor cells have been reported to migrate into central nervous system injury sites [54–56] and bone-forming sites [57], respectively, the tropism of injected BM cells toward diabetic nerves has not been previously documented, although another study by our group showed similar neurotropism of i.m. injected BM-derived EPCs [45]. In this study, we have shown that SDF-1 $\alpha$  and MCP-1, which are highly expressed in diabetic nerves, mediate these neurotropic effects in part. However, further detailed study is required to fully elucidate the molecular mechanisms underlying this neurovascular tropism to diabetic nerves, and these studies are under way by our group.



However, our study has limitations that must be addressed before it can be readily extrapolated to human clinical settings. First, the duration of diabetes and the follow-up period after cell transplantation is shorter than those of human patients. Given the recent report by our group showing that transplantation of BM-derived EPCs improved and maintained the nerve function in diabetic mice at least up to 8 weeks after cell transplantation [45], it is very likely that the therapeutic effects of BMNC transplantation would last longer than 4 weeks. However, because the life expectancy of DN patients is much longer than that of animal models, it should be determined whether the improved nerve function can be maintained for years or longer. Second, the therapeutic effects of the patient's own diabetic BMNCs in comparison to normal BMNCs need to be further evaluated because of a potential detrimental influence of diabetes on BM cells. On the positive side, we did not find any adverse effects of BMNC treatment. In fact, BMNCs have been used in various clinical trials and have been proven to be safe for administration into many organs including heart, ischemic limb, and liver [58–61]. For clinical studies, the proposed dose for human patients will be around 250 million cells, because the body surface area of humans is about 50 times larger than that of rats and the therapeutic dose is known to be linearly proportional to the body surface area. In addition, as the sciatic nerve is about 8–10 times longer in human patients, about 30–50 injections will be required to treat each DN patient, as used in a previous clinical study using BMNCs for human ischemic limb patients [34]. The proven safety and the therapeutic effects shown by our study render BMNC treatment an excellent candidate for a clinical trial to treat DN.

## Conclusion

*i.m.* transplanted BMNCs preferentially localized to diabetic nerves, especially around the vasa nervorum. These BMNCs increased various angiogenic and neurotrophic factors in the nerves, leading to increased vascularity and restoration of neural function in DN. Taken together, these novel findings suggest that BMNC therapy can be an innovative therapeutic modality for DN patients.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

This work was supported in part by NIH grants (HL079137, HL084471), a Juvenile Diabetic Research Foundation Innovation Grant (5–2007-951), and a grant (SC4300) from the Stem Cell Research Center of the 21st Century Frontier Research Program funded by the Ministry of Science and Technology, Republic of Korea. The authors have no conflicting financial interests. J.S.P. is currently affiliated with the Division of Cardiology, Department of Internal Medicine, Yeungnam University Hospital, South Korea. M.I. is currently affiliated with the Group of Vascular Regeneration, Institute of Biomedical Research and Innovation, Kobe, Japan.

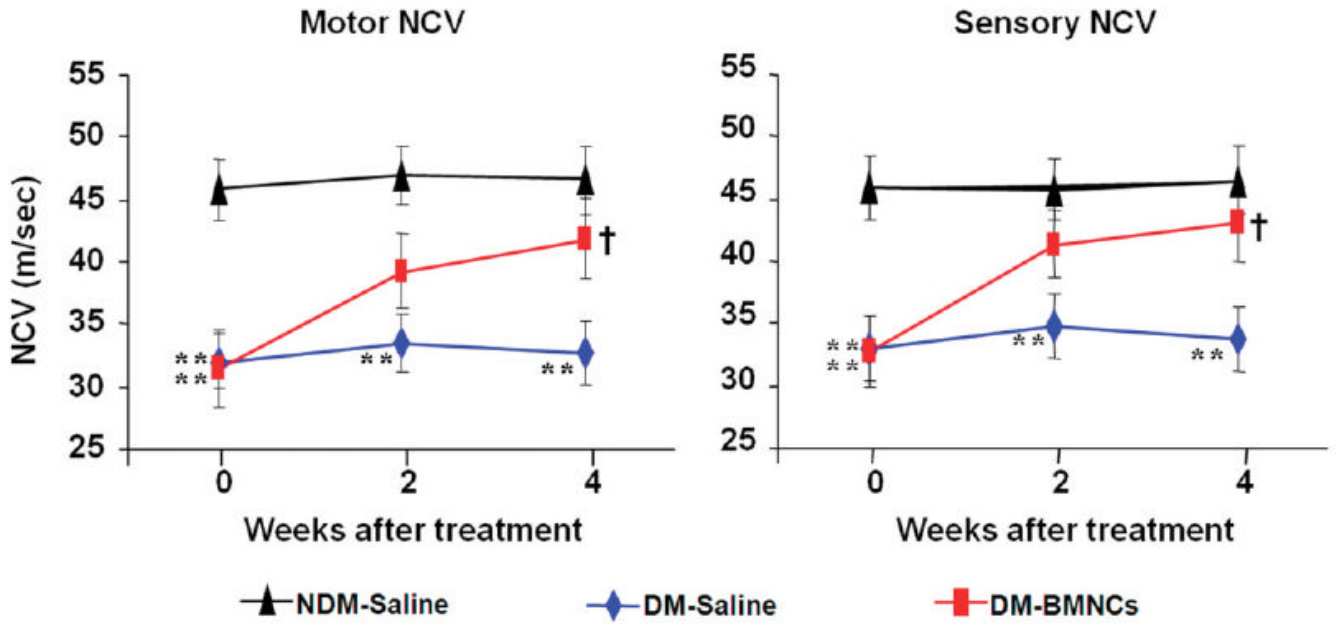
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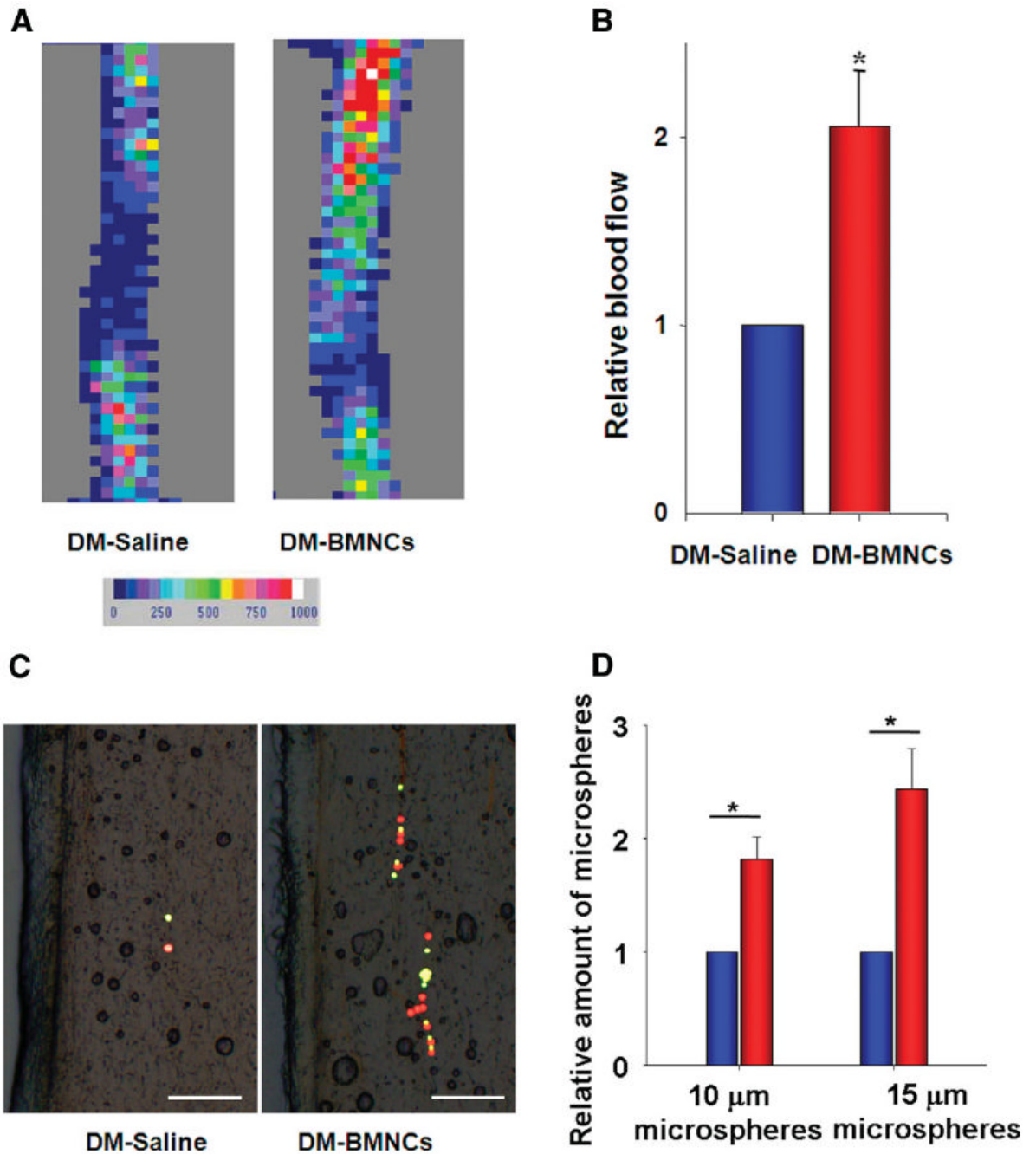
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**Figure 1.**

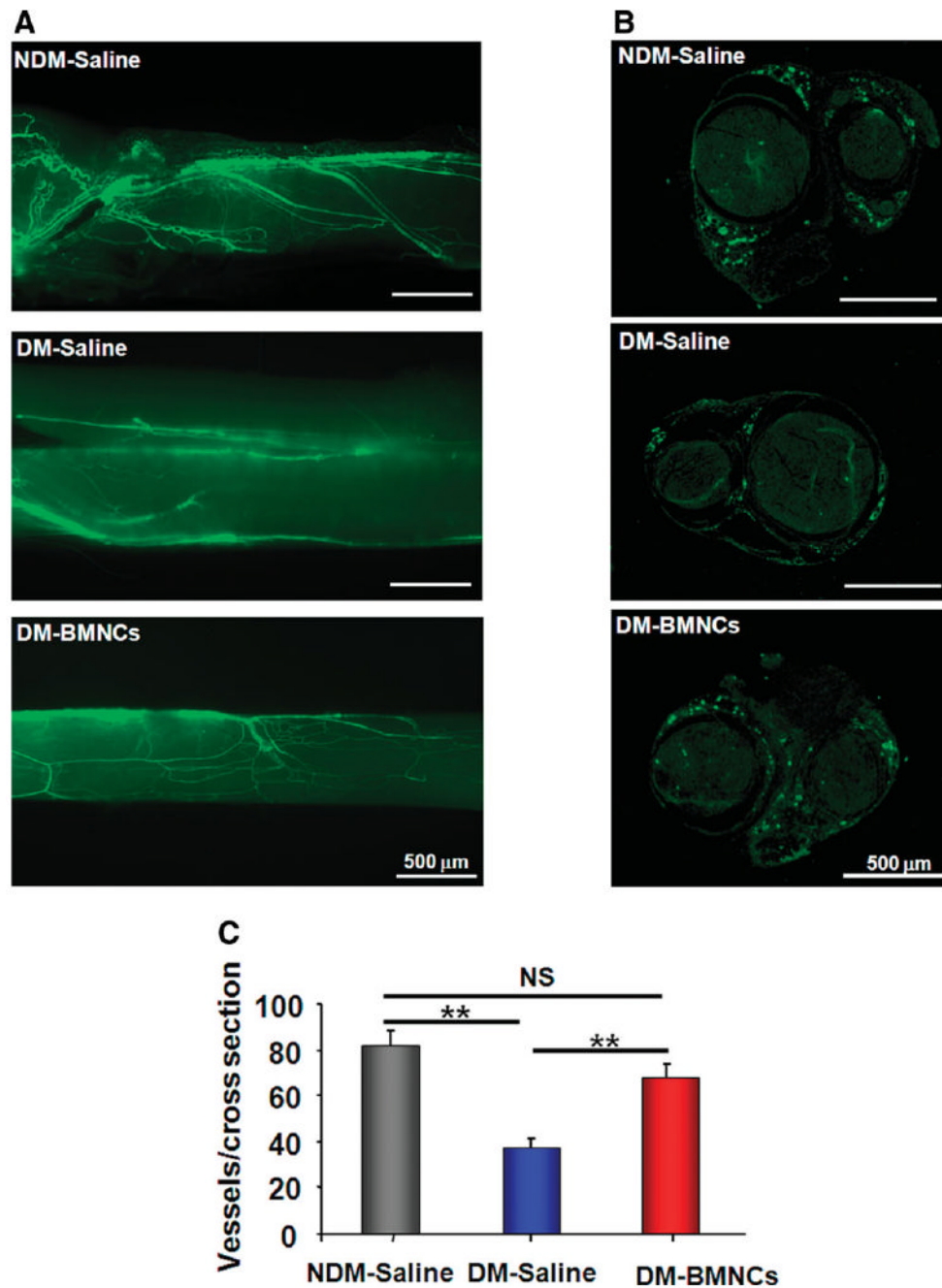
NCVs in diabetic nerves were improved by BMNC injection. Twelve weeks after streptozotocin injection, both motor and sensory NCVs in diabetic rats were significantly lower than in age-matched nondiabetic rats (NDM-Saline versus DM-Saline: \*\*,  $p < .01$ ;  $n = 10$  each group). BMNC injection significantly improved both motor and sensory NCVs in diabetic rats over 4 weeks (DM-Saline versus DM-BMNCs: †,  $p < .05$ ;  $n = 10$  each group). Abbreviations: BMNC, bone marrow-derived mononuclear cell; DM-BMNCs, diabetic rats treated with BMNCs; DM-Saline, diabetic rats treated with saline; NCV, nerve conduction velocity; NDM-Saline, nondiabetic rats treated with saline.



**Figure 2.**

BMNC transplantation increased blood perfusion in diabetic nerves. (A): Representative images of blood perfusion taken by LDPI show greater blood flow in a diabetic rat injected with BMNCs than in the saline-injected one. The amount of blood flow is color-coded: lowest blood flow is indicated by dark blue and highest blood flow by red and white. (B): Quantitative evaluation of LDPI demonstrates that BMNC transplantation significantly improved blood perfusion in diabetic nerves compared with saline injection (DM-Saline versus DM-BMNCs: \*,  $p < .05$ ;  $n = 7$ ). (C): Representative images of blood flow determined using fluorescent microspheres. Fluorescent images were merged with bright-field images of the nerve tissue. Higher numbers of 10- $\mu$ m green and 15- $\mu$ m red microspheres were observed in the BMNC-

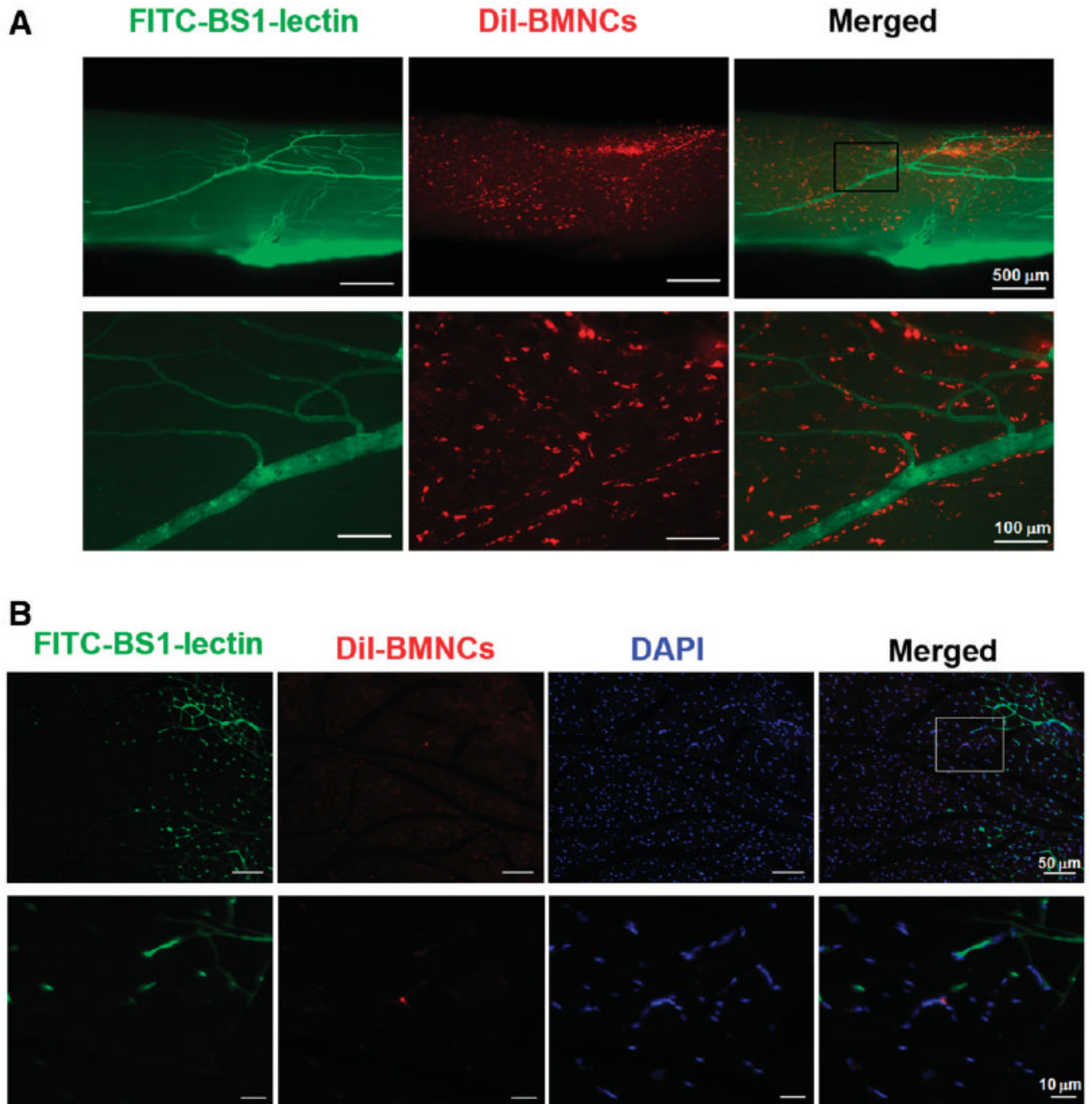
treated group than in saline controls. Bars, 200  $\mu\text{m}$ . **(D)**: Quantitation of the microspheres in the nerve corroborates that BMNC transplantation significantly improved blood flow in the diabetic nerves compared with saline control (\*,  $p < .05$ ;  $n = 4$ ). Abbreviations: BMNC, bone marrow–derived mononuclear cell; DM-BMNCs, diabetic rats treated with BMNCs; DM-Saline, diabetic rats treated with saline; LDPI, laser Doppler perfusion imager.



**Figure 3.** BMNC transplantation replenished vascularity in diabetic nerves. To visualize functional blood vessels, rat hindlimbs were perfused with FITC-conjugated BS-1 lectin 4 weeks after BMNC transplantation. In both longitudinal whole-mount (A) and cross-sectional (B) images of the sciatic nerves, vasa nervorum, represented by green fluorescence, was observed more abundantly in nondiabetic rats than in diabetic control rats injected with saline. Diabetic nerves treated with saline showed a marked decrease in small branches of large epineurial blood vessels, which were restored by BMNC transplantation. Quantitative evaluation of blood vessels in the cross-sections of the nerves (C) confirmed a significant increase in the number of vasa nervorum in diabetic nerves treated with BMNCs over those treated with saline (NDM-

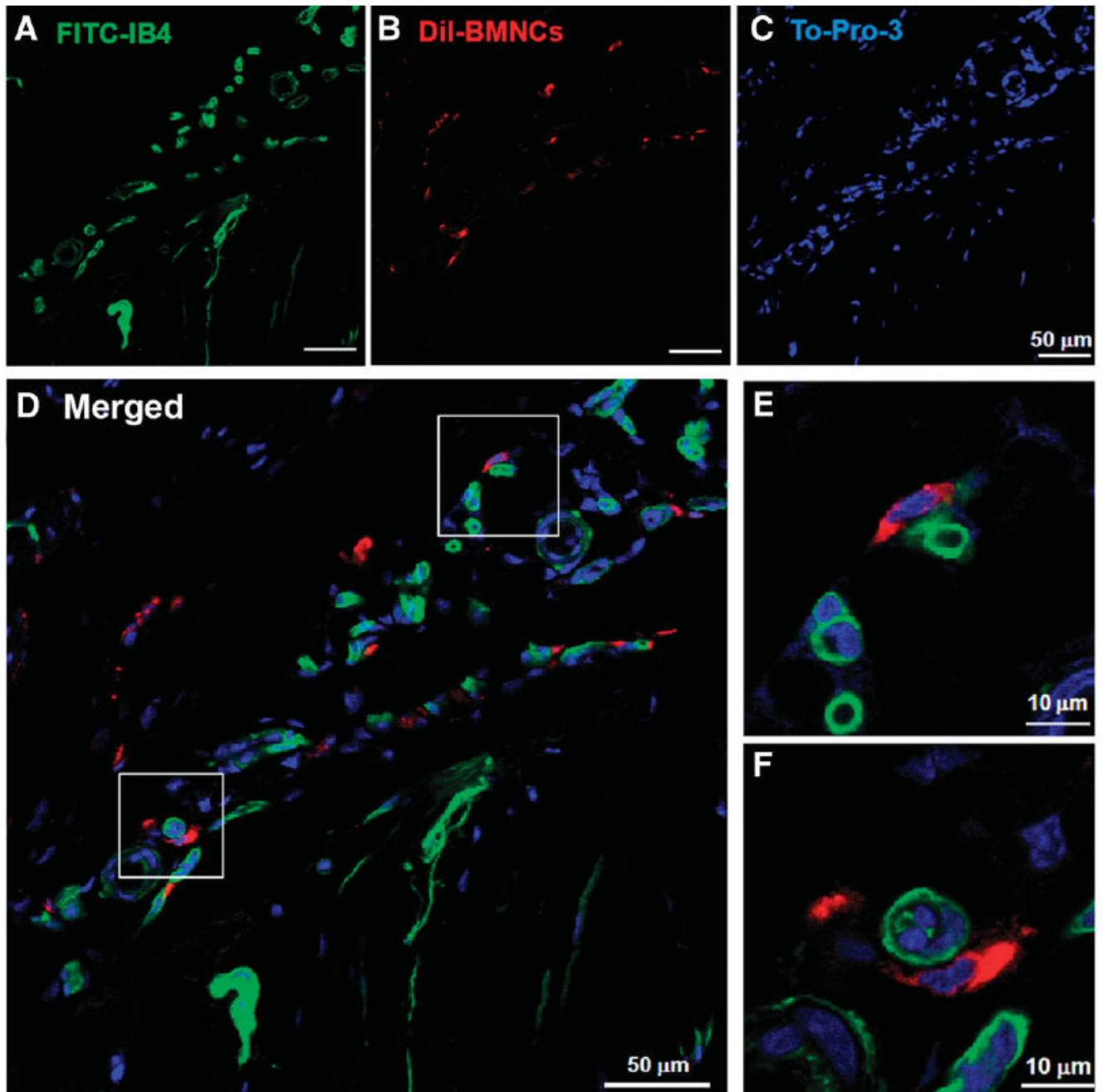


Saline versus DM-Saline: \*\*,  $p < .01$ ; DM-Saline versus DM-BMNCs: \*\*,  $p < .01$ ;  $n = 5$  each group). Bars,  $500 \mu\text{m}$ . Abbreviations: BMNC, bone marrow–derived mononuclear cell; DM-BMNCs, diabetic rats treated with BMNCs; DM-Saline, diabetic rats treated with saline; FITC, fluorescein isothiocyanate; NDM-Saline, non-diabetic rats treated with saline.



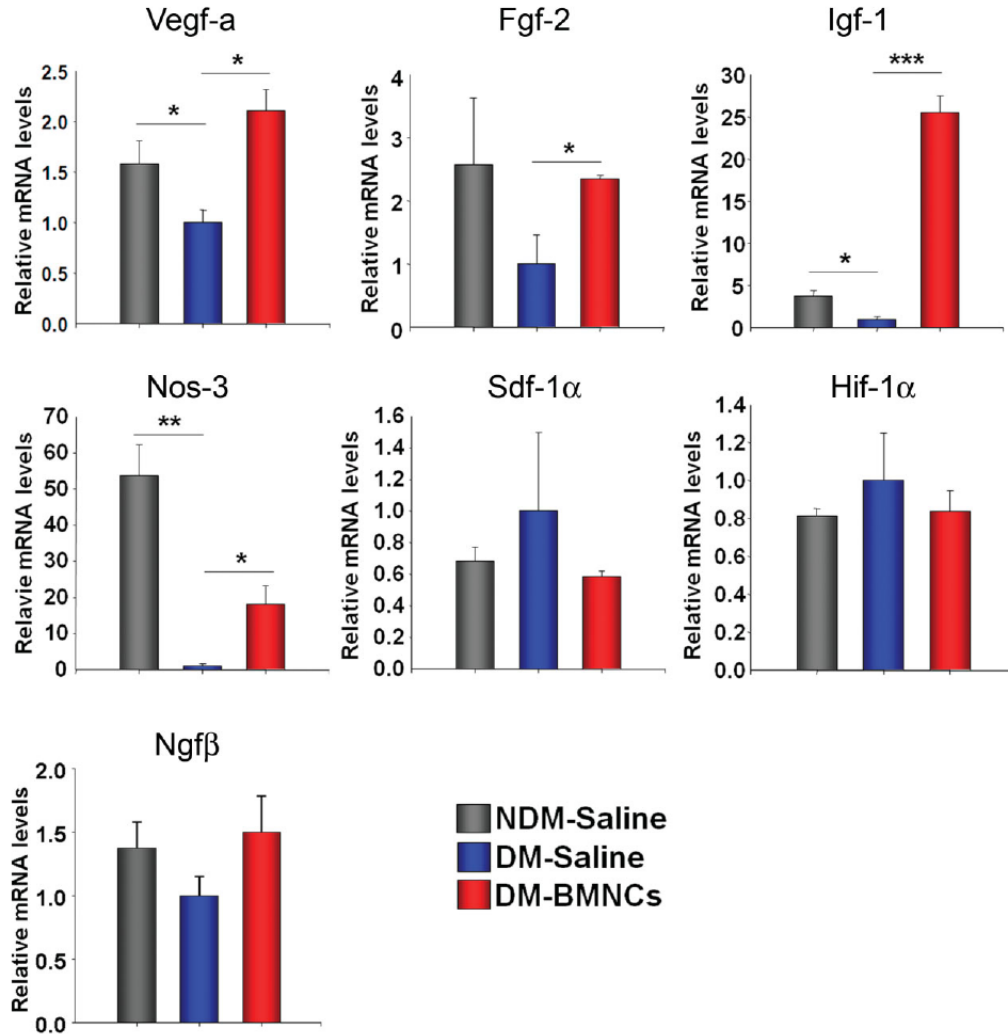
**Figure 4.**

Transplanted BMNCs localized mainly to sciatic nerves along the vasa nervorum. Hindlimbs were perfused with FITC-conjugated BS-1 lectin (green fluorescence) to visualize blood vessels before harvesting nerve and muscle tissue 4 weeks after DiI-labeled BMNC (red fluorescence) injection. **(A)**: Whole-mount longitudinal images revealed robust engraftment of DiI-labeled BMNCs in the diabetic nerves mainly along the vasa nervorum. **(B)**: DiI-labeled BMNCs were less frequently observed in the muscle tissue under fluorescent microscopy. **(A, B)**: Bottom panels show higher magnification images of the boxed regions in the top panels. Abbreviations: BMNC, bone marrow-derived mononuclear cell; DAPI, 4',6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate.



**Figure 5.**

Transplanted BMNCs do not incorporate as ECs at detectable levels in diabetic nerves. Shown are representative cross-sectional confocal images of diabetic nerves 4 weeks after injection of DiI-labeled BMNCs. Blood vessels and nuclei were identified with FITC-conjugated isolectin B4 and To-Pro-3 staining, respectively. A large portion of injected BMNCs were in close contact with ECs; however, exact colocalization with ECs was not observed, suggesting that the injected BMNCs do not transdifferentiate into, nor fuse with, ECs at a detectable frequency. **(D)**: Merged image of **(A)**, **(B)**, and **(C)**. **(E, F)**: Higher magnification images of the boxed region in **(D)**. Abbreviations: BMNC, bone marrow–derived mononuclear cell; EC, endothelial cell; FITC, fluorescein isothiocyanate.



**Figure 6.** Effect of BMNC injection on the level of angiogenic and neurotrophic factors. Two weeks after injection of BMNCs, total RNA was isolated from the sciatic nerves. The levels of mRNA of angiogenic and neurotrophic factors were determined using real-time RT-PCR and normalized to GAPDH. The levels of Vegf-a, Fgf-2, Igf-1, and Nos-3 mRNA were significantly higher in diabetic nerves treated with BMNC than in those treated with saline (\*,  $p < .05$ ; \*\*,  $p < .01$ ; \*\*\*,  $p < .001$ ;  $n = 3-7$ ). All values were normalized to the mRNA level of each factor in the DM-Saline group. Abbreviations: BMNC, bone marrow-derived mononuclear cell; DM-BMNCs, diabetic rats treated with BMNCs; DM-Saline, diabetic rats treated with saline; Fgf-2, fibroblast growth factor-2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Hif-1α, hypoxia-inducible factor-1α; Igf-1: insulin-like growth factor-1; NDM-Saline, nondiabetic rats treated with saline; Ngfβ, nerve growth factorβ; Nos-3, endothelial nitric oxide synthase; RT-PCR, reverse transcription-polymerase chain reaction; Sdf-1α; stromal cell-derived factor-1α; Vegf-a, vascular endothelial growth factor-A.

**Table 1**

Sequences of primers and probes for quantitative reverse transcription-polymerase chain reaction

mRNA	Forward primer (5'-3')	Reverse primer	Probe
Vegf-a	GCGGGCTGCTGCAATG	CATAGTGACGTTGCTCTCCGAC	AGCCCTGGAGTGCGTGCCCA
Fgf-2	AAGGATCCCAAGCGGCTCTA	CGGCCGTCTGGATGGA	ACGGCGGCTTCTTCCTGCGC
Igf-1	CCTACAAAGTCAGCTCGTTCCA	GGGCTGGGACTTCTGAGTCTT	CGGGCCCAGCGCCACACT
Nos-3	CACAAGAGTTACAAAATCCGATTCA	TCTGTGTTACTGGATTCTTCCTTT	CCACTGGTATCCTCTTGGCGGCG
Sdf-1a	CTGGGCACAGTTACAGGTGGT	TGCTCTGGTGGAAGGTTGCT	CTCCCCAGGGCTCCCAGCAAA
Hif-1a	GATCAGTTGTCACCATTAGAGAGCAA	TGGGTCTGCTGGAATCCTGTA	CCAAGCCCTCCGAGTGTGAGCAC
Ngfb	CACTGTGGACCCCAAACTGTTT	GGTGGAGGCTGGGTGCTA	AAACGGAGACTCCGTTACCCCG
Gapdh	CCGAGGGCCCACTAAAGG	TGCTGTTGAAGTCACAGGAGACA	CATCCTGGGCTACACTGAGGACCAGG

Abbreviations: Fgf-2, fibroblast growth factor-2; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Hif-1 $\alpha$ , hypoxia-inducible factor-1 $\alpha$ ; Igf-1: insulin-like growth factor-1; Ngfb, nerve growth factor $\beta$ ; Nos-3, endothelial nitric oxide synthase; Sdf-1 $\alpha$ ; stromal cell-derived factor-1 $\alpha$ ; Vegf-a, vascular endothelial growth factor-A.