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Dual Angiogenic and Neurotrophic Effects of Bone Marrow–Derived Endothelial Progenitor Cells on Diabetic Neuropathy

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Abstract

Background—Endothelial progenitor cells (EPCs) are known to promote neovascularization in ischemic diseases. Recent evidence suggested that diabetic neuropathy is causally related to impaired angiogenesis and deficient growth factors. Accordingly, we investigated whether diabetic neuropathy could be reversed by local transplantation of EPCs.

Methods and Results—We found that motor and sensory nerve conduction velocities, blood flow, and capillary density were reduced in sciatic nerves of streptozotocin-induced diabetic mice but recovered to normal levels after hind-limb injection of bone marrow–derived EPCs. Injected EPCs were preferentially and durably engrafted in the sciatic nerves. A portion of engrafted EPCs were uniquely localized in close proximity to vasa nervorum, and a smaller portion of these EPCs were colocalized with endothelial cells. Multiple angiogenic and neurotrophic factors were significantly increased in the EPC-injected nerves. These dual angiogenic and neurotrophic effects of EPCs were confirmed by higher proliferation of Schwann cells and endothelial cells cultured in EPC-conditioned media.

Conclusions—We demonstrate for the first time that bone marrow-derived EPCs could reverse various manifestations of diabetic neuropathy. These therapeutic effects were mediated by direct augmentation of neovascularization in peripheral nerves through long-term and preferential engraftment of EPCs in nerves and particularly vasa nervorum and their paracrine effects. These

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findings suggest that EPC transplantation could represent an innovative therapeutic option for treating diabetic neuropathy.

Keywords

angiogenesis; diabetes mellitus; progenitor cells; diabetic neuropathy

Peripheral neuropathy is the most common complication of diabetes mellitus, affecting up to 60% of diabetic patients.[1] Loss of sensation in the feet, the most frequent manifestation of diabetic neuropathy (DN), frequently leads to foot ulcers and may progress into amputation of the limb.[2,3] Despite a continuous increase in the incidence of diabetes mellitus and DN, current treatments have yet to effectively treat DN. Our group reported that experimental DN is characterized by reduced microcirculation in peripheral nerves caused by the destruction of the vasa nervorum and thus administration of angiogenic factors such as vascular endothelial growth factors (VEGFs), sonic hedgehog (SHh), and statin could restore neural function by augmenting angiogenesis.[4–6] In addition, deficiency of neurotrophic factors is regarded as one of the most plausible mechanisms underlying DN.[7] Alterations of nerve growth factor, ciliary neurotrophic factor, glial-derived neurotrophic factor, and brain-derived neurotrophic factor have been reported.[8–12] However, in clinical trials, single neurotrophic cytokines turned out to be ineffective for treating DN.[13] Recently, many classic angiogenic factors were shown to possess neurotrophic activities and vice versa. VEGF,[14–16] SHh,[17,18] insulin-like growth factor-1,[19] and neurotrophins[20,21] are some of the representative factors with these dual effects. Because DN lacks both angiogenic and neurotrophic factors, using a therapeutic agent that has dual angioneurotrophic activities may prove more beneficial for treating DN. In this regard, endothelial progenitor cells (EPCs) can be an optimal candidate for treating DN because they possess paracrine properties that encompass both angiogenic and neurotrophic effects. Furthermore, unlike protein or gene therapy, cell therapy may be able to provide long-term effects. EPCs are putative progenitor cells of endothelial cells, exist in peripheral blood and bone marrow (BM), and contribute to postnatal neovascularization. Growing evidence suggests that EPCs are effective in treating various cardiovascular diseases.[22–25] Mechanistically, EPCs work through transdifferentiation into vasculature[22,26] and paracrine effects.[24,27] The paracrine effects are made possible because EPCs produce multiple biological factors such as VEGF, insulin-like growth factor-1, and fibroblast growth factor-2 (FGF-2). Accordingly, we sought to investigate whether transplantation of EPCs could attenuate or reverse DN by augmenting neovascularization and providing angiogenic and neurotrophic factors.

In the present study, we report that BM-derived EPCs, by directly augmenting neural neovascularization, could effectively treat DN. We found for the first time that intramuscularly injected EPCs are preferentially engrafted into peripheral nerves, are specifically localized around vasa nervorum, and increase the expression of various angiogenic and neurotrophic factors.

Methods

Induction of Diabetes Mellitus

All protocols were approved by St Elizabeth's Institutional Animal Care and Use Committee. We induced diabetes mellitus in 6-week-old male C57BL/6J mice by intraperitoneal injection of streptozotocin (150 mg/kg).

Isolation of EPCs and Cell Culture

For EPC culture, the bones of 8-week-old C57BL/6J mice were excised and crushed with PBS. Mononuclear cells were fractionated by density gradient centrifugation. Isolated mononuclear cells were cultured on rat plasma vitronectin–precoated 10-cm dishes.[23,24]

Intramuscular Injection of Cultured EPCs

We labeled EPCs with a red fluorescent dye, CM-DiI (Invitrogen, Carlsbad, Calif), as previously described[28] and injected EPCs (1×10^6) or the same volume of saline into the muscles percutaneously along the course of the sciatic nerve.

Laser Doppler Imaging of Vasa Nervorum Blood Flow

Perfusion of sciatic vasa nervorum was measured in each hind limb of mice with a laser Doppler perfusion imager (Moor Instruments, Millwey, Axminster, Devon, UK).[4,5] After anesthesia, nerves were exposed, and flow measurements were repeated twice over the same region of interest.

Fluorescent Imaging of Blood Vessels in Sciatic Nerves and Femoral Muscles

Vascularity of sciatic nerves and femoral muscles was assessed by in situ fluorescent staining with an endothelial cell–specific marker, BS-1 lectin.[4,5] After anesthesia, the hind limbs were perfused with BS-1 lectin conjugated to FITC (Vector Laboratories, Burlingame, Calif) by cardiac injection. Fifteen minutes later, the animals were killed, and the sciatic nerves and femoral muscles were harvested. After fixation, samples were either whole mounted or embedded in optical coherence tomography compound for frozen section.

Statistical Analysis

All results are presented as mean ± SEM. Statistical analysis was performed by an unpaired Student *t* test for comparisons between 2 groups and ANOVA for >2 groups. For statistical analysis of nerve conduction velocity (NCV) measurements in Figure 1, we used a repeated-measures ANOVA. Values of $P<0.05$ are considered statistically significant.

Details on the materials and methods, including the following items, can be found in the online-only Data Supplement: measurements of NCV,[6,15] tail-flick testing,[4,6] double-fluorescence immunohistochemistry for BrdU[28] and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL),[29] in vitro cell proliferation assay,[6] quantitative reverse-transcription polymerase chain reaction for mRNA expression,[24] and Western blot analysis.[29]

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

EPC Transplantation Improves Neural Function in Diabetic Mice

To determine the impact of local transplantation of EPCs on the function of peripheral nerves in diabetes mellitus, we measured NCVs every 2 weeks for 8 weeks after treatment. At baseline, 12 weeks after induction of diabetes mellitus, both motor and sensory NCVs were slowed by ≈35% and 40%, respectively, in diabetic mice (injected with saline) compared with the nondiabetic mice (NDM-saline), indicating development of significant peripheral neuropathy (Figure 1A and 1B). Diabetic mice were randomly assigned to EPC (DM-EPC) or saline injection (DM-saline) groups and were injected intramuscularly around the sciatic nerves. After EPC treatment, both motor and sensory NCVs gradually recovered to normal levels over 8 weeks (Figure 1A and 1B). Statistically, a repeated-measures ANOVA demonstrated a

significant difference in NCVs between the DM-EPC and DM-saline groups at baseline ($P<0.05$) and at 4 and 8 weeks ($P<0.01$). Tail-flick testing 4 weeks after treatment showed that in DM-saline mice, tail-flick temperatures were significantly increased compared with nondiabetic control mice. In contrast, in the DM-EPC mice, tail-flick temperatures were significantly decreased to the level of nondiabetic mice, indicating recovery of sensory nerve function (Figure 1C).

EPC Transplantation Improves Neural Vascularity in Diabetic Mice

We measured sciatic nerve blood flow in each limb using laser Doppler flow imaging at 4 weeks after treatment. Nerve blood flow was markedly decreased in DM-saline compared with NDM-saline mice ($P<0.05$; Figure 2A and 2B). The blood flow and the blood flow ratio between treated and nontreated limbs were significantly increased in the DM-EPC mice compared with the DM-saline mice ($P<0.001$; Figure 2C).

Next, to investigate changes in functional vessels, we harvested sciatic nerves at 4 weeks after treatment after injection of BS-1 lectin into the heart. Whole-mount images of sciatic nerves showed that DM-saline mice had fewer functional vasa nervorum, which are responsible for perfusion of peripheral nerves, compared with NDM-saline mice ($P<0.01$) (Figure 3A and 3B, left and middle). In contrast, the vasa nervorum was visibly increased after EPC treatment (Figure 3A and 3B, middle and right). Quantitative analysis of the vessels in cross sections of sciatic nerves showed a higher number of vasa nervorum in the EPC-injected mice compared with the saline-injected mice (Figure 3C).

Transplanted EPCs Home to Sciatic Nerves and Are Durably Engrafted

Next, we studied the engraftment and transdifferentiation characteristics of EPCs in sciatic nerves harvested at 2, 4, 8, and 12 weeks after EPC transplantation. The endothelial characteristics of cultured EPCs were confirmed by conventional EPC assay and fluorescence-activated cell sorter analyses. We found that 31% of the EPCs were positive for Tie2 expression and 91% for CD11b expression, suggesting that a majority of cultured EPCs originated from a monocyte population and that cultured EPCs possess proangiogenic/vasculogenic potential (Figure I of the online-only Data Supplement).[27] To identify vasa nervorum, we injected BS-1 lectin before the mice were killed. In whole-mount preparations of sciatic nerves, we observed that intramuscularly injected EPCs, prelabeled with the red fluorescent dye DiI, homed to sciatic nerves and robustly engrafted over the study period of 12 weeks (Figure 4A and 4B). In contrast, engrafted EPCs were observed less frequently in the femoral muscles at 2 weeks, and most of the EPCs disappeared within 8 weeks (Figure 4C). These findings suggest that EPCs preferentially home to the sciatic nerve and that a large number of the engrafted EPCs migrated along the course of and abutting the vasa nervorum (Figure 5A through 5C). Immunohistochemistry and fluorescence-activated cell sorter analysis of the digested nerves at 8 weeks after treatment revealed that ≈90% of the engrafted EPCs exhibit CD11b and Tie2, suggestive of the phenotype of proangiogenic Tie2-expressing monocytes/macrophages (online-only Data Supplement Figure IIA and IIB).[30] In cross sections, a small portion of the EPCs expressed an endothelial cell phenotype, suggesting transdifferentiation into endothelial cells (Figure 5D and 5E). These data show that locally injected EPCs have specific tissue tropism to diabetic nerves and affinity for endothelial cells within the nerves. We found that the expression of SDF-1α and MCP-1 was detected only in the nerves, not in the muscles, which suggests that these factors may play a role in recruiting EPCs to diabetic nerves (online-only Data Supplement Figure III).

EPC Transplantation Increases Proliferation of Endothelial Cells and Schwann Cells

We also investigated the paracrine effects of EPCs. We implanted a mini-osmotic pump loaded with BrdU in the back skin immediately after treatment that released BrdU for 4 weeks.[28] These

sciatic nerves were harvested at 4 weeks, and double immunohistochemistry with antibodies against BrdU and a Schwann cell marker, S-100, was performed. We found >4 times as many BrdU-positive Schwann cells in the DM-EPC group as in the DM-saline group (Figure 6A and 6B). To determine whether this proproliferative effect could be mediated through the paracrine action of EPCs, we used hypoxic conditions to mimic the in vivo status of diabetic nerves that are under ischemia as a result of the loss of vasa nervorum. Schwann cells or endothelial cells (human umbilical vein endothelial cells [HUVECs]) were cultured in EPC-conditioned media, collected after EPCs had been cultivated either in normoxia or 5% hypoxia or in plain EBM-2 (Figure 6C and 6D). We found that proliferation of both Schwann cells and HUVECs grown in hypoxic EPC-conditioned media was significantly higher than controls (Schwann cells, 13.4% increase over 3% FBS, $P<0.01$; HUVECs, 24.4% increase over 3% FBS, $P<0.01$; Figure 6C and 6D). Taken together, these findings suggest that transplanted EPCs effectively induced proliferation of Schwann cells and endothelial cells through their paracrine activity.

EPC Transplantation Decreases Apoptosis in Endothelial Cells and Schwann Cells in Diabetic Nerves

We further asked whether EPC transplantation can affect ongoing apoptosis in diabetic nerves. We performed TUNEL assay on sciatic nerves obtained 1 week after cell transplantation. The number of TUNEL-positive cells was 4-fold higher in the nerves of DM-saline mice than in NDM-saline mice ($P<0.05$; Figure 7A and 7B), but this number was reduced by 50% in the DM-EPC mice ($P<0.001$ versus DM-saline; Figure 7B). Qualitatively, concomitant staining with TUNEL and either ILB4 or S100 revealed that apoptosis occurred in both endothelial cells and Schwann cells (Figure 7C and 7D).

EPC Transplantation Increases Multiple Angiogenic, Antiapoptotic, and Neurotrophic Factors

Next, to determine whether paracrine factors secreted by EPCs could mediate the proproliferative and antiapoptotic effects of EPCs, we examined the levels of angiogenic and neurotrophic factors in sciatic nerves at 4 weeks after EPC transplantation. Quantitative reverse-transcription polymerase chain reaction revealed that mRNA expression levels of angiogenic and neurotrophic factors were higher in the EPC-injected group than in the saline-injected group (VEGF-A, 3.4±1.2-fold; FGF-2, 1.5±0.3-fold; and Gli 1, 2.6±0.8-fold, all $P<0.05$; brain-derived neurotrophic factor, 5.9±1.2-fold; SHh, 2.4±0.4-fold; and SDF-1α, 1.9 ±0.1-fold, all $P<0.001$; Figure 8A through 8G). The levels of nerve growth factor, angiopoietin 1, epidermal growth factor, and hepatocyte growth factor were too low to be detected (data not shown). Western blot analysis further demonstrated that the protein levels of VEGF, FGF-2, and Gli 1 were significantly increased in the EPC group compared with the saline group (VEGF A, 2.2±0.5-fold; FGF-2, 1.6±0.2-fold; and Gli 1, 1.9±0.3-fold, all $P<0.05$). These findings suggest that intramuscular injection of EPCs upregulated multiple angiogenic and neurotrophic factors at the mRNA and protein levels (Figure 8H and 8I).

Discussion

In this study, we have found that local transplantation of BM-derived EPCs improved various manifestations of experimental DN through direct effects on peripheral nerves. Some of the most salient findings of the present study are as follows. First, local transplantation of BM-derived EPCs restored neurophysiological deficits in DN. Second, EPC treatment increased neural vascularity functionally and histologically. Third, intramuscularly injected EPCs preferentially homed to sciatic nerves, characteristically localized in close proximity to vasa nervorum, and transdifferentiated into endothelial cells, albeit infrequently. Fourth, a large number of engrafted EPCs survived in peripheral nerves for >12 weeks and induced prolonged

expression of angiogenic and neurotrophic factors. Fifth, EPC transplantation increased proliferation and decreased apoptosis of endothelial and Schwann cells.

It is our hypothesis that microvascular insufficiency in nerves plays a major role in the development and progression of DN and therefore that therapeutic intervention by EPC transplantation can reverse or attenuate DN by inducing neovascularization and supplying angioneurotrophic cytokines. The coincidence of restoration of vasa nervorum accompanied by functional nerve recovery has been documented in DN with distinct angiogenic agents.[4–6] Again, this study provides strong evidence that the development of and recovery from DN are pathophysiologically associated with loss and gain, respectively, of vasa nervorum.

The most notable finding of the present study is the direct effect of EPCs on peripheral nerves. BS-1 lectin perfusion experiments clearly demonstrate for the first time that EPC transplantation increases capillary density and blood flow in nerves, suggesting that EPCs induce genuine neovascularization in nerves. Mechanistically, because transdifferentiation of EPCs into endothelial cells was observed only infrequently, our results suggest a greater contribution by angiogenesis than vasculogenesis to this process. This neural angiogenesis appears to be made possible through upregulation of various angiogenic factors in nerves after EPC transplantation. In fact, this is the first evidence documenting upregulation of multiple paracrine or humoral factors in peripheral nerves after treatment with stem/progenitor cells. In this study, factors such as VEGF-A,[4,15] FGF-2,[31] brain-derived neurotrophic factor,[32] SHh,[5,18] and SDF-1α,[33,34] which are known to function as both angiogenic and neurotrophic factors, were highly expressed in EPC-transplanted nerves. These upregulated factors could have provided additional benefits for the recovery of neural function by promoting proliferation and inhibiting apoptosis of Schwann cells. In addition, these humoral effects might be contributed not only by the injected EPCs but also by the recovering nerves after EPC treatment.[24] This is the first report showing such dual angiogenic and neurotrophic effects of EPCs. This upregulation of various classes of biologically important factors may be one of the greatest benefits of stem cell therapy over any single protein or gene therapy, enabling the concerted efforts of multiple neuroangiogenic cytokines necessary for neurovascular recovery.

One prior study showed that cord blood– derived EPCs were effective for treating DN.[35] Although this study reported a therapeutic potential of cord blood cells, mechanistically, a wide difference exists between that study and the present one. The study by Naruse et al,[35] which used umbilical cord blood–derived EPCs, suggested that the therapeutic effects might be due to increased differentiation of EPCs into endothelial cells in hind-limb muscles, which then led to an increase in sciatic nerve blood flow. However, that study did not investigate the fate or engraftment characteristics of the EPCs in tissues, nor did it address the mechanisms by which transplanted EPCs increase neovascularization in muscles or nerve. In fact, more studies argue against the transdifferentiation of EPCs as a major mechanism underlying therapeutic effects.[24,27] In contrast, our study clearly provided 2 important mechanistic insights. First, intramuscularly injected EPCs exert therapeutic effects through direct modulation of nerves, not through muscular neovascularization. Second, the dominant mechanism is humoral or paracrine effects, not transdifferentiation. Histological examination of our samples revealed that in hind-limb muscles the number of engrafted EPCs was much smaller, and a majority of EPCs disappeared within 8 weeks; in the sciatic nerves, however, EPCs robustly survived for >12 weeks. Interestingly, the study by Naruse et al[35] showed that capillary density, which had decreased in hind-limb muscles of diabetic rats at 12 weeks of diabetes mellitus, was significantly increased after cord blood EPC treatment. In contrast, our data suggested that blood flow and capillary density were decreased mildly but not statistically significantly in hind-limb muscles. This discrepancy might have been due to the difference in animal species or genetic backgrounds of mice[20] used for a diabetic model: Naruse et al used nude rats, whereas we used C57BL6/J mice. Nude rats exhibit more severe impairment in angiogenesis

because they lack T cells[36]; this blunted angiogenic response in the nude rat might have caused the significantly reduced vascularity seen in the hind-limb muscle in diabetic nude rat. Thus, we have concerns that using human cord blood EPCs in nude rats might not have properly addressed the mechanisms involving the therapeutic effects of EPCs resulting from xenogenic mismatch and the choice of an angiogenically impaired animal model. In contrast, the present study, by using syngeneic mice for both donors and recipients, avoided such potential confounding factors.

We made 3 novel observations on the fate of EPCs in tissues. One of the most striking findings was that EPCs homed to peripheral nerves far more preferentially than to muscles (Figure 5). This scale of close interaction between any BM cells and steady-state tissues was not previously reported either with or without diabetes mellitus. SDF-1α and MCP-1 produced by diabetic nerves seemed to be able to attract injected EPCs. Another notable finding was the durable engraftment of BM-derived EPCs into diabetic nerves. After reports on the short-lasting engraftment of transplanted BM cells in a myocardial infarction model,[37,38] the notion has been widely accepted that engrafted adult stem/progenitor cells disappear within a couple of weeks. However, the present study disclosed that a large number of BM-derived progenitor cells could survive for a prolonged period of time, 12 weeks, in nerves. These data indicate that the engraftment characteristics of progenitor cells may depend more on the recipient environment than on the transplanted cells themselves. However, a limitation of this study is that although we detected long-term EPC engraftment, the 12-week time frame is much shorter than the clinical course of this disease. The last intriguing finding is that the engrafted EPCs were localized in close proximity to the vasa nervorum. To the best of our knowledge, such a significant magnitude of tropism of BM-derived cells to blood vessels has not been reported in any other tissues, either in normal or in diseased states. These unique characteristics of BM-derived EPCs, ie, peripheral neurotropism, sustained engraftment, and vascular localization of EPCs, could have caused robust and prolonged paracrine or humoral effects and led to the reversal of functional and histological impairment of peripheral nerves in diabetes mellitus.

Because advanced DN, which is a likely candidate for cell therapy, is frequently combined with and presents by diabetic foot ulcers and/or limb ischemia and because EPCs are also known to be effective for treating diabetic wounds or lower-limb ischemia, a therapeutic approach of using EPCs in advanced DN can be clinically relevant and valuable. Practically, because the safety of autologous BM-derived EPCs or similar progenitor cells has been documented by a number of clinical trials,[25,39] it would be possible to advance this strategy into a pilot clinical trial. The effectiveness of the patient's own diabetic EPCs versus healthy EPCs needs to be evaluated because of a potential concern about the negative effects of diabetes mellitus on EPCs. Taken together, these findings suggest that cell therapy with BM-derived EPCs may represent an innovative therapeutic option for treating DN.

Clinical Perspective

In the United States alone, >18 million people suffer from diabetes mellitus. Peripheral neuropathy is the most common complication of diabetes mellitus, affecting up to 60% of long-standing diabetic patients. Diabetic neuropathy (DN) commonly manifests with loss of sensation in the feet, frequently leading to foot problems such as ulcers. Despite a continuous increase in the incidence of diabetes and DN, current treatments have yet to effectively treat DN. Recent evidence suggests that DN is causally related to impaired angiogenesis and deficient neurotrophic factors. Endothelial progenitor cells exist in peripheral blood and bone marrow. Therapeutically, preclinical and clinical pilot studies have demonstrated that endothelial progenitor cells are effective in repairing various cardiovascular diseases via differentiation into new vessels and production of angiogenic

and neurotrophic factors. In this study, we demonstrate that local injection of endothelial progenitor cells reversed functional impairments of DN in experimental DN by augmenting neovascularization and providing angiogenic and neurotrophic factors in diabetic nerves. Our study suggests a novel therapeutic strategy, the application of stem/progenitor cell therapy, for DN and provides new insight into the pathophysiological features of DN. Considering that DN is frequently combined with diabetic foot ulcers, limb ischemia, or both, an approach that uses endothelial progenitor cells could have additional clinical benefits for treating complicated DN.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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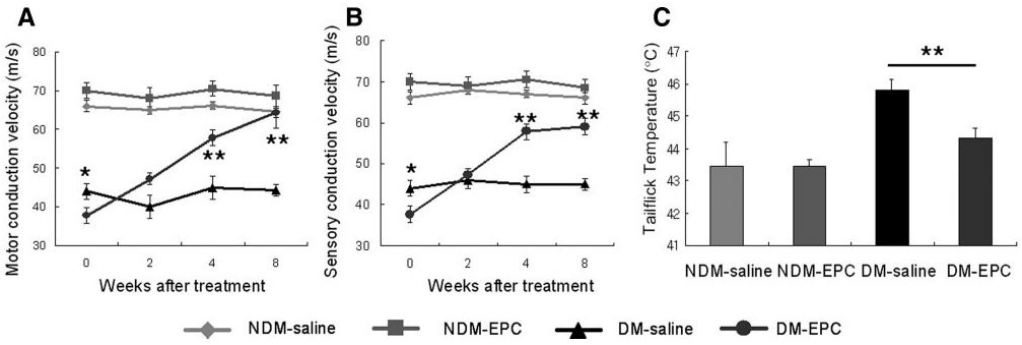

Figure 1.

EPC transplantation improved nerve function in diabetic mice. A and B, Twelve weeks after streptozotocin injection, DM-saline mice developed severe peripheral neuropathy represented by significant reductions in both motor and sensory NCVs (**P*<0.05, NDM-saline vs DM-saline). NCVs were significantly improved over 8 weeks after EPC transplantation (***P*<0.01, DM-EPC vs DM-saline). C, A tail-flick test was performed 4 weeks after EPC transplantation. In DM-saline mice, the tail-flick temperature was increased significantly (**P*<0.05, NDM-saline vs DM-saline). In DM-EPC mice, the tail-flick temperature decreased to the level of nondiabetic control mice, suggesting recovery of sensory function (***P*<0.05, DM-EPC vs DM-saline). n=11 each group.

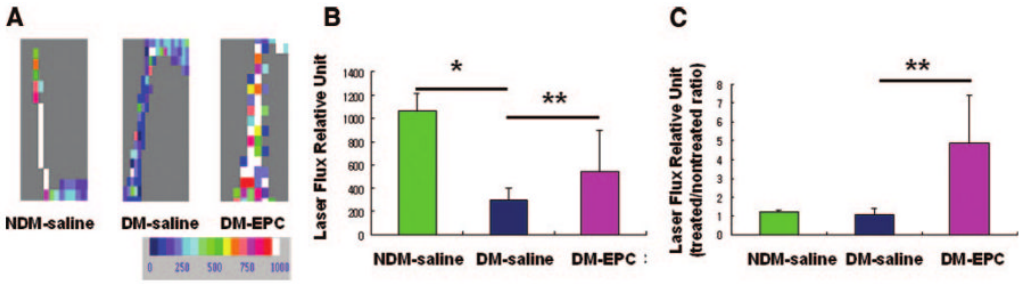

Figure 2.

EPC transplantation increased blood perfusion in diabetic nerves. A, Laser Doppler flow imaging of sciatic nerves showed markedly decreased blood flow in the diabetic nerve vs the nondiabetic nerve (**P*<0.05, NDM-saline vs DM-saline). B and C, Quantitative evaluation by laser flux unit (B) and the ratio of laser flux unit (treated/nontreated limb) (C) at 4 weeks after treatment showed a significant increase in blood flow in the sciatic nerve in the EPC group vs the control group (***P*<0.001, DM-EPC vs DM-saline). n=6 each group.

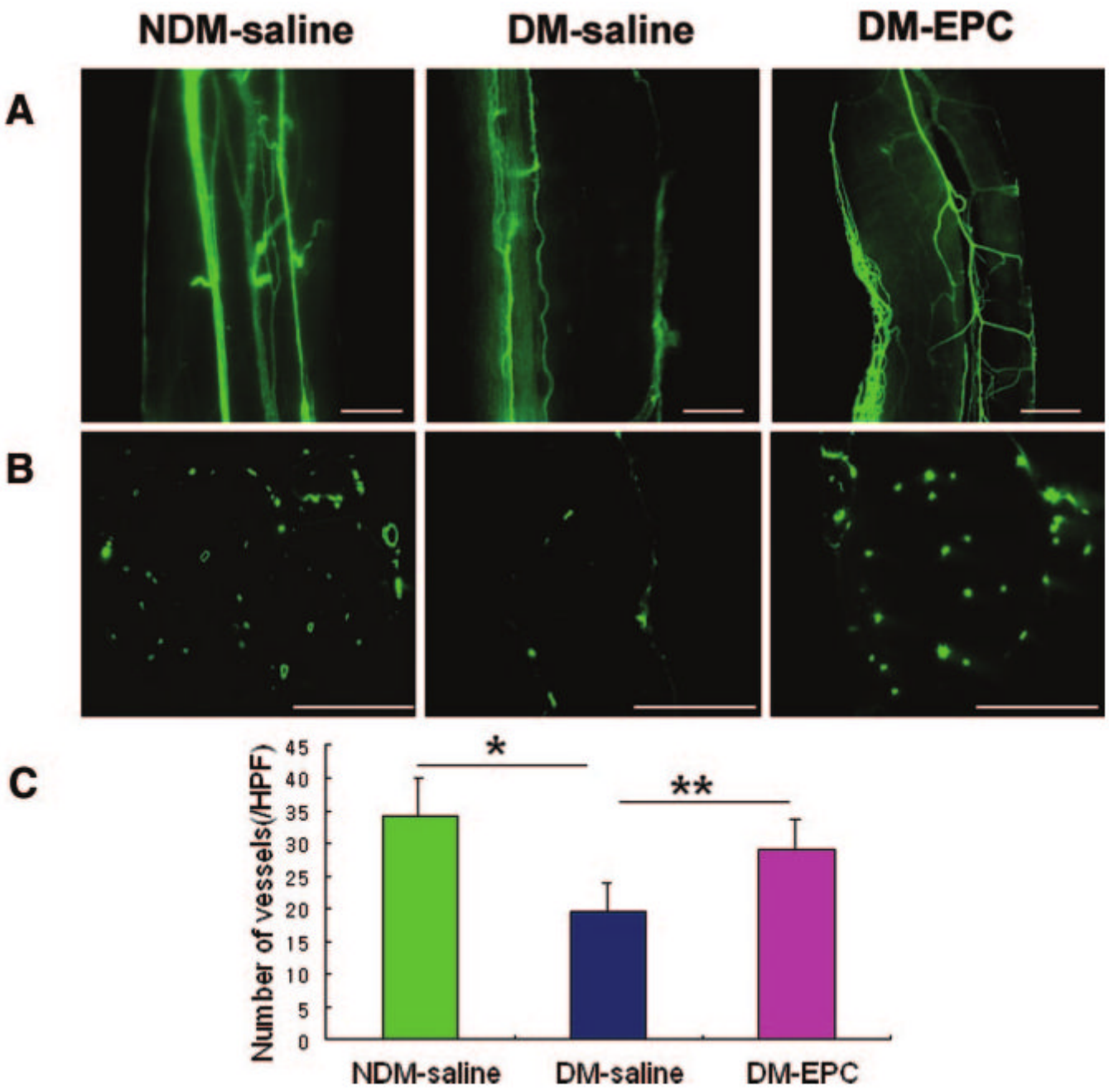

Figure 3.

EPC transplantation restored neural vascularity in DN. We injected BS-1 lectin to perfuse blood vessels and obtained whole-mount images of sciatic nerves. In both longitudinal (A) and cross (B) sections of the sciatic nerves, BS-1 lectin–stained vasa nervorum (green fluorescence) was seen more abundantly in nondiabetic mice and DM-EPC mice compared with diabetic control (saline) mice. Quantitative analysis in cross sections (C) showed a higher number of vasa nervorum in the EPC-injected diabetic mice vs the saline-injected diabetic mice. (*P<0.01, NDM-saline vs DM-saline; **P<0.05, DM-EPC vs DM-saline). Bars=100 μm. n=10 each group.

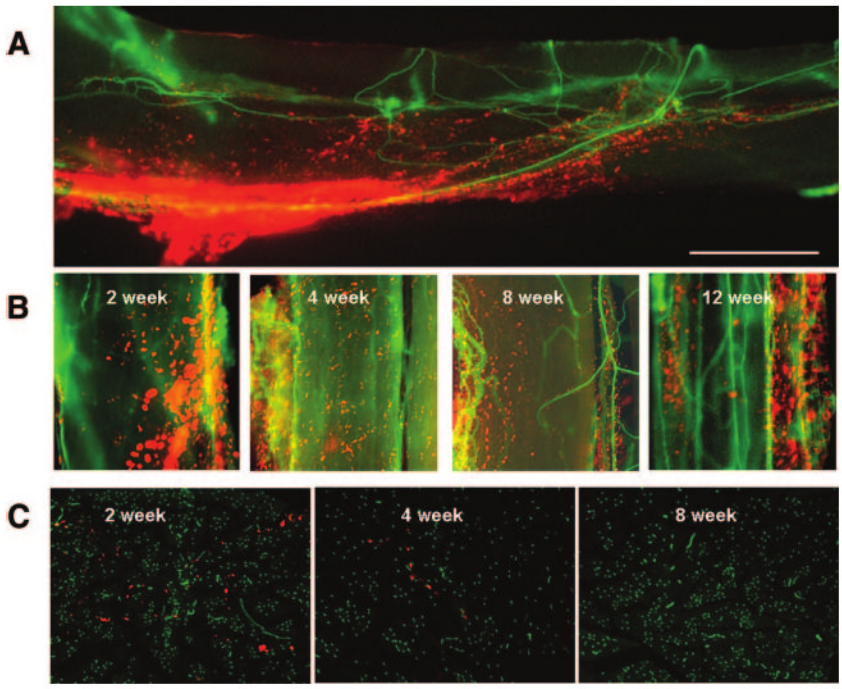

Figure 4.

Preferential and sustained engraftment of transplanted EPCs in sciatic nerves. A, A representative whole-mount image of a sciatic nerve from a diabetic mouse after perfusion with BS-1 lectin (green) demonstrated robust engraftment of DiI-labeled EPCs (red) into sciatic nerves at 4 weeks after EPC transplantation. Bars=500 μm. B, Serial images of diabetic nerves after EPC transplantation showed sustained engraftment of EPCs over 12 weeks. Bars=100 μm. C, Fluorescent microscopic images of femoral muscles harvested from the same mice shown in B demonstrated that most of the transplanted EPCs (red fluorescence) disappeared within 8 weeks. Bars=100 μm.

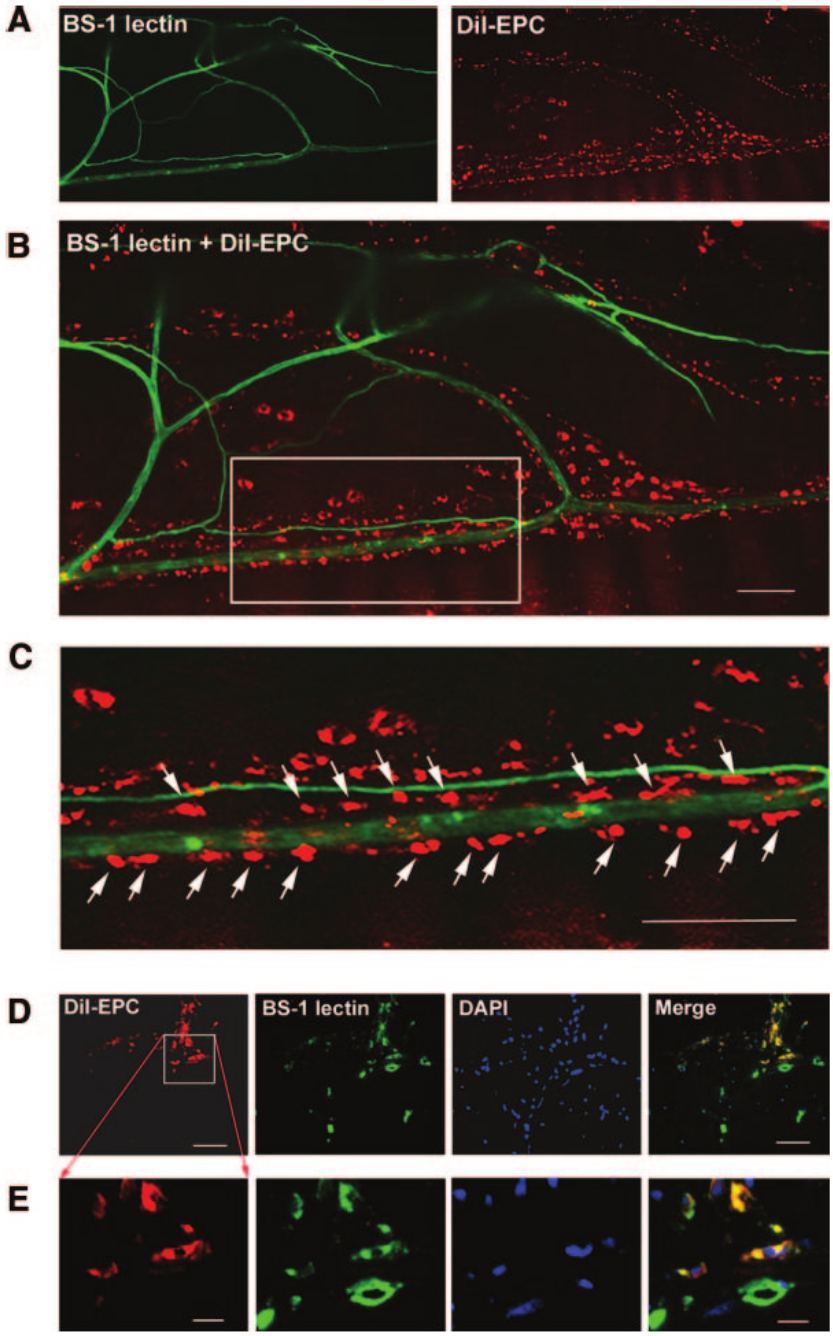

Figure 5.
Localization of transplanted EPCs along the vasa nervorum and transdifferentiation of EPCs into endothelial cells in nerves. A, B, and C, Whole-mount images of a sciatic nerve demonstrated that engrafted EPCs (red) were preferentially localized along the course of the vasa nervorum (green). B and C, Bars=50 μm. D and E, Cross-sectional images of a diabetic nerve 4 weeks after injection with EPCs revealed that a portion of the engrafted EPCs was colocalized with BS-1 lectin–positive endothelial cells suggestive of transdifferentiation of EPCs into endothelial cells. Bars=50 μm (D) and 10 μm (E).

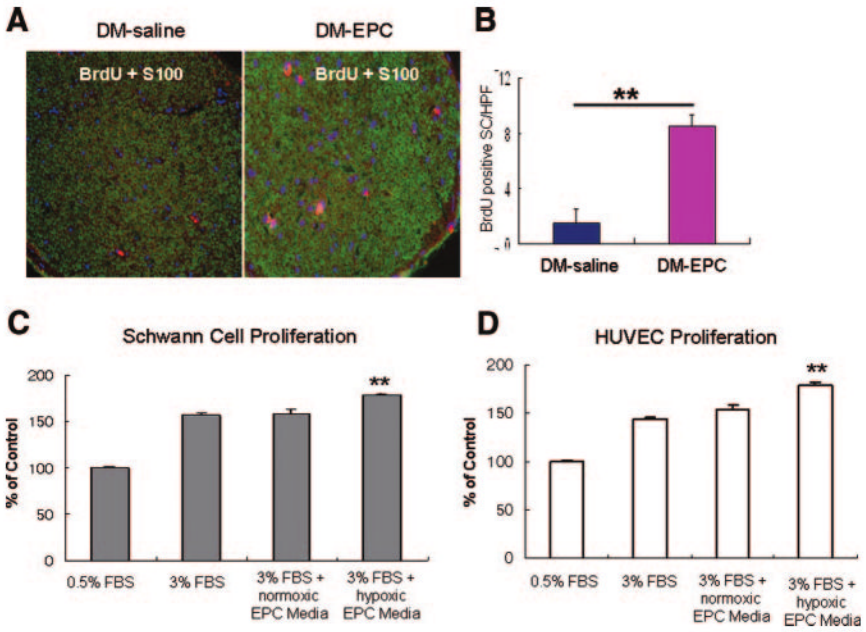

Figure 6.

EPC transplantation increased Schwann cell and endothelial cell proliferation. A and B, Double immunohistochemistry with antibodies against BrdU (red) and S-100 (green) demonstrated significantly higher numbers of BrdU-positive Schwann cells in the EPC-transplanted nerves than in the saline-injected nerves at 4 weeks. ***P*<0.05. Bars=100 μm. Blue fluorescence is DAPI. n=4 each group. C and D, Schwann cells (C) and HUVECs (D) were cultured in the following culture conditions: EBM-2 media containing 0.5% FBS, EBM-2 media including 3% FBS, EBM-2 media containing 3% FBS in which EPCs were cultured under normoxia (3% FBS+normoxic EPC media), and EBM-2 media containing 3% FBS in which EPCs were cultured under 5% O^2 (3% FBS+hypoxic EPC media). Both Schwann cells and HUVECs grown in hypoxic EPC-conditioned media proliferate significantly more than controls (Schwann cells: 13.4% increase over 3% FBS group, *P*<0.01 vs 3% FBS; HUVECS: 24.4% increase over 3% FBS group, *P*<0.01 vs 3% FBS). n=8 each group.

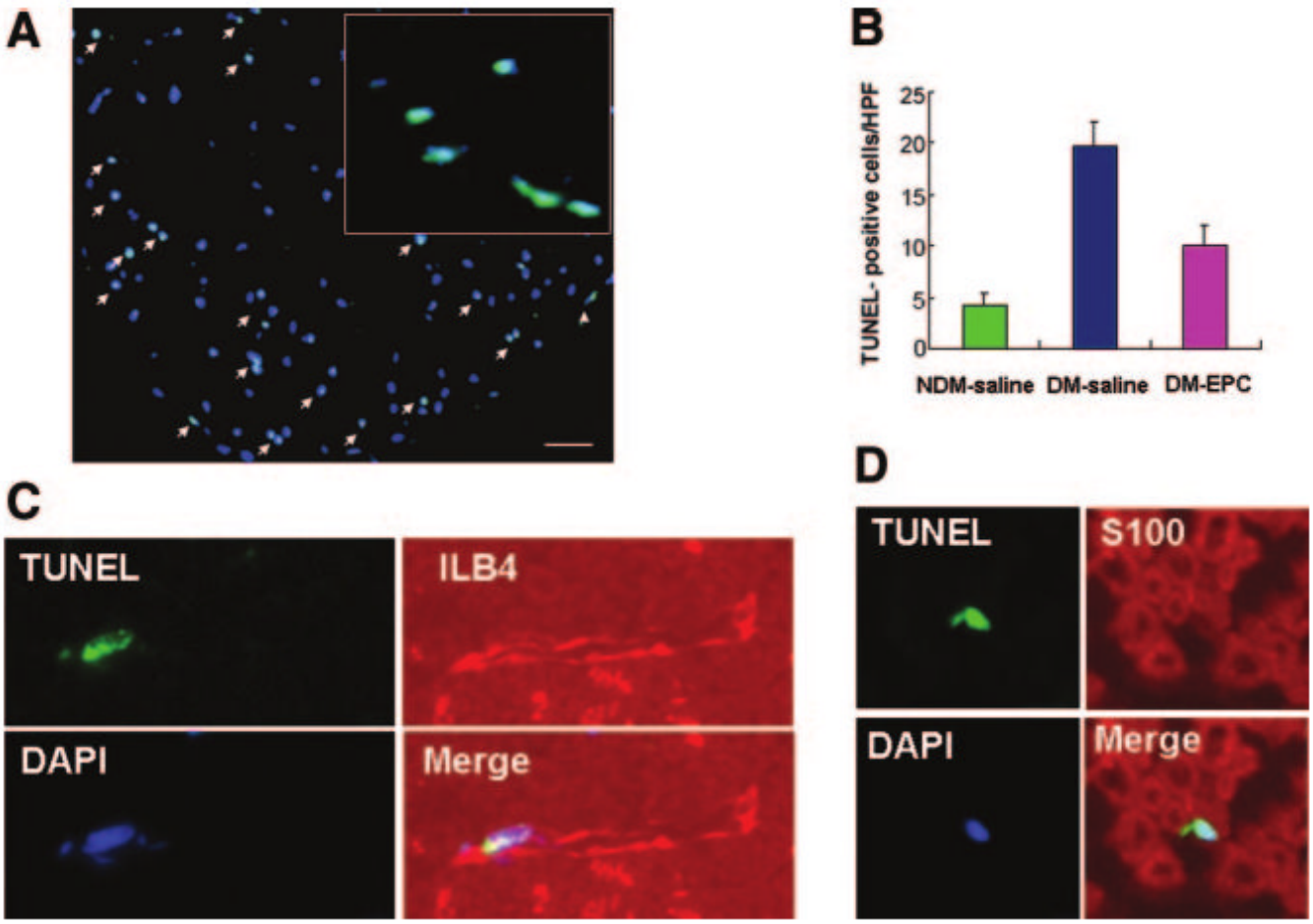

Figure 7.

EPC transplantation decreased apoptosis. A, A representation illustrating TUNEL-positive cells (green fluorescence) in a diabetic nerve. Inset shows the magnified view of TUNEL- and DAPI-positive nuclei in a diabetic nerve. Blue is DAPI. Bar=50 μm. B, The number of TUNEL-positive cells was 4-fold higher in diabetic nerves than in nondiabetic nerves (*P<0.05, DM-saline vs NDM-saline). EPC-transplanted diabetic nerves have reduced TUNEL-positive cells (**P<0.001, DM-saline vs DM-EPC). n=5 each group. C and D, To further investigate the identity of TUNEL-positive cells, double immunohistochemistry was performed. Both ILB4-positive endothelial cells (C) and S100-positive Schwann cells (D) showed positive reaction in TUNEL assay. Bars=20 μm. HPF indicates high-power field.

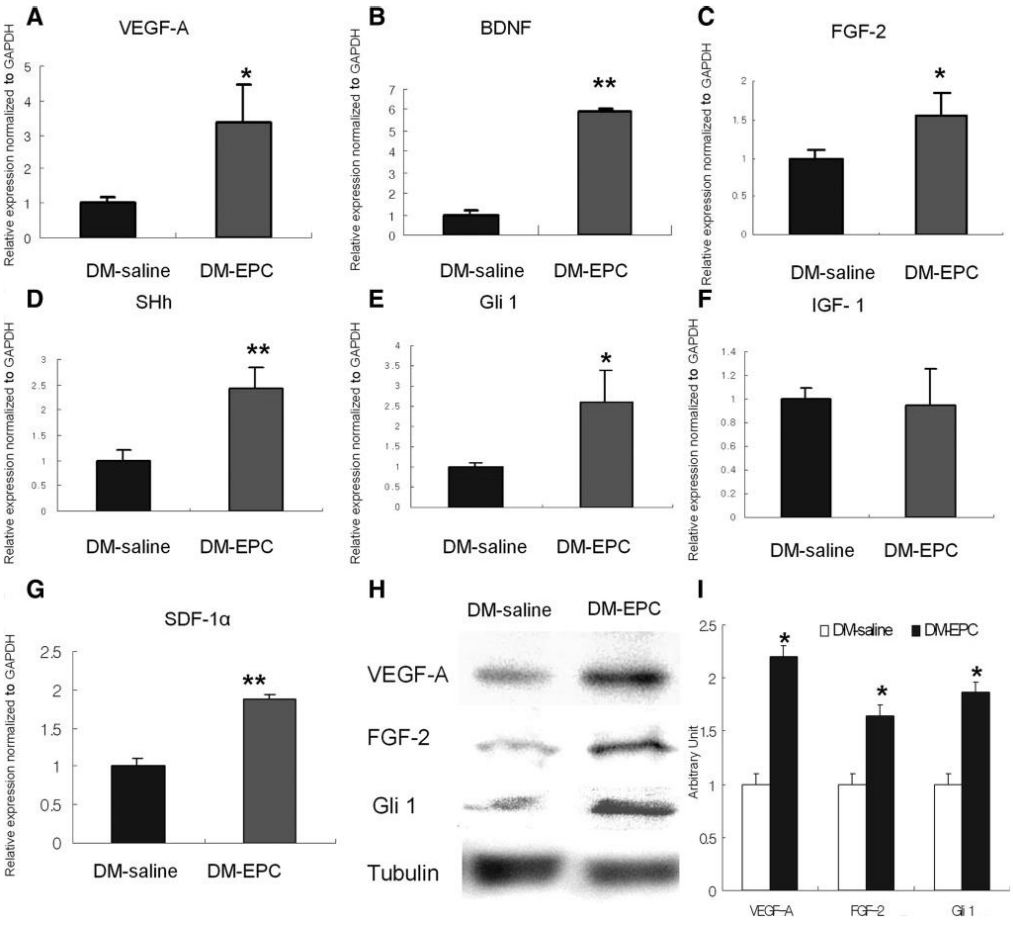

Figure 8.

EPC transplantation increased multiple angiogenic and neurotrophic factors. A through G, Real-time reverse-transcription polymerase chain reaction demonstrated that expression levels of VEGF-A, brain-derived neurotrophic factor (BDNF), FGF-2, SHh, Gli 1, and SDF-1*α* were significantly increased in sciatic nerves from the EPC-transplanted group vs the saline-injected group (**P*<0.05). H and I, Western blot analysis of sciatic nerves further confirmed that the protein expression of VEGF-A, FGF-2, and Gli 1 was significantly increased in the EPC group vs the saline group. n=12 each group.