J Neurosurg 116:432–444, 2012

Recruitment by SDF-1α of CD34-positive cells involved in sciatic nerve regeneration

Laboratory investigation

Meei-Ling Sheu, Ph.D.,[1] Fu-Chou Cheng, Ph.D.,[4,6] Hong-Lin Su, Ph.D.,[2] Ying-Ju Chen, Ph.D.,[4] Chun-Jung Chen, Ph.D.,[6] Chih-Ming Chiang, M.S.,[5] Wen-Ta Chiu, M.D., Ph.D.,[7] Jason Sheehan, M.D., Ph.D.,[8] and Hung-Chuan Pan, M.D., Ph.D.[1,3]

Institutes of [1]Biomedical Sciences and [2]Life Sciences, National Chung-Hsing University; Departments of [3]Neurosurgery, [5]Radiology, and [6]Education and Medical Research, and [4]Stem Cell Center, Taichung Veterans General Hospital, Taichung; [7]Department of Neurosurgery, Taipei Medical University–Shuang Ho Hospital, Taipei Medical University, Taipei, Taiwan; and [8]Department of Neurosurgery, University of Virginia Health System, Charlottesville, Virginia

Object. Increased integration of CD34+ cells in injured nerve significantly promotes nerve regeneration, but this effect can be counteracted by limited migration and short survival of CD34+ cells. SDF-1α and its receptor mediate the recruitment of CD34+ cells involved in the repair mechanism of several neurological diseases. In this study, the authors investigate the potentiation of CD34+ cell recruitment triggered by SDF-1α and the involvement of CD34+ cells in peripheral nerve regeneration.

Methods. Peripheral nerve injury was induced in 147 Sprague-Dawley rats by crushing the left sciatic nerve with a vessel clamp. The animals were allocated to 3 groups: Group 1, crush injury (controls); Group 2, crush injury and local application of SDF-1α recombinant proteins; and Group 3, crush injury and local application of SDF-1α antibody. Electrophysiological studies and assessment of regeneration markers were conducted at 4 weeks after injury; neurobehavioral studies were conducted at 1, 2, 3, and 4 weeks after injury. The expression of SDF-1α, accumulation of CD34+ cells, immune cells, and angiogenesis factors in injured nerves were evaluated at 1, 3, 7, 10, 14, 21, and 28 days after injury.

Results. Application of SDF-1α increased the migration of CD34+ cells in vitro, and this effect was dose dependent. Crush injury induced the expression of SDF-1α, with a peak of 10–14 days postinjury, and this increased expression of SDF-1α paralleled the deposition of CD34+ cells, expression of VEGF, and expression of neurofilament. These effects were further enhanced by the administration of SDF-1α recombinant protein and abolished by administration of SDF-1α antibody. Furthermore, these effects were consistent with improvement in measures of neurological function such as sciatic function index, electrophysiological parameters, muscle weight, and myelination of regenerative nerve.

Conclusions. Expression of SDF-1α facilitates recruitment of CD34+ cells in peripheral nerve injury. The increased deposition of CD34+ cells paralleled significant expression of angiogenesis factors and was consistent with improvement of neurological function. Utilization of SDF-1α for enhancing the recruitment of CD34+ cells involved in peripheral nerve regeneration may be considered as an alternative treatment strategy in peripheral nerve disorders. *(DOI: 10.3171/2011.3.JNS101582)*

Key Words • **hematopoietic progenitor cell** • **SDF-1α** • **nerve regeneration** • **sciatic nerve crush injury** • **peripheral nerve**

Several approaches have been proposed to facilitate beneficial effects of peripheral nerve regeneration, including application of an electric field, transplantation of stem cells, and administration of neurotrophic factors.[5,29,33,35,37] Cell replacement, trophic factor production, ECM synthesis, guidance of ECM synthesis, axonal growth cone guidance, remyelination, microenvironmental stabilization, and immune modulation have been postulated as possible mechanisms for cell implantation.[8,29,43] Transplantation of whole-bone marrow cells, bone marrow–derived hematopoietic stem cells, bone

Abbreviations used in this paper: CMAP = compound muscle action potential; ECM = extracellular matrix; G-CSF = granulocyte colony-stimulating factor; HPC = hematopoietic progenitor cell; RI = regularity index; SFI = sciatic function index.

This article contains some figures that are displayed in color online but in black and white in the print edition.

marrow–derived mesenchymal stem cells, or bone marrow–derived endothelial progenitor cells has been demonstrated to promote functional recovery in injured peripheral nerves.[3,9,12,15] In addition, mobilization of bone marrow progenitor cells by G-CSF has been shown to facilitate nerve regeneration mostly through deposits of $CD34^+$ cells, which paralleled significant expression of angiogenesis factors.[50] Hence, through either endogenous mobilization or exogenous supplementation, HPCs significantly enhance nerve regeneration and should be considered as part of a treatment strategy.

Bone marrow contains populations of precursor cells that can differentiate into a number of cell types, including bone, cartilage, and mesenchymal stem cells.[38] Bone marrow cells have been shown to induce angiogenesis and to produce several trophic factors during this process, making them potential candidates for use in neural regeneration.[36] Furthermore, transplantation of untreated bone marrow cells, bone marrow–derived hematopoietic stem cells, bone marrow–derived mesenchymal stem cells, or bone marrow–derived endothelial progenitor cells has become a therapeutic strategy in the treatment of peripheral nerve disease. The postulated mechanisms are attributed to cell differentiation, trophic factor secretion, and modulation of glial and neuronal cell populations.[3,9,12,15,39] In addition, the mobilization of bone marrow cells, especially $CD34^+$ cells, by G-CSF is also believed to exert an influence on the injured nerve to promote nerve regeneration. These beneficial effects of $CD34^+$ cell deposition have been shown to be highly correlated with VEGF expression and vascularization/angiogenesis.[34]

Recruitment of HPCs from the bone marrow into peripheral blood following treatment with chemotherapy and/or cytokines is termed "mobilization." The release of HPCs from the bone marrow is a physiological phenomenon that protects them from toxic injury. Circulating cells can reenter bone marrow, thereby maintaining a fixed number of HPCs in bone marrow.[1] Bone marrow cells also enter the peripheral vascular system in response to stress signals during injury and inflammation of hematopoietic and nonhematopoietic tissues.[20,22,26] As mobilized peripheral blood stem cells are increasingly used clinically for cell transplantation, it is becoming clear that proteolytic degradation of chemokines and of SDF-1α and its receptor on stem cells (CXCR-4) could be an important step in stem cell release and homing.[23] Furthermore, administration of G-CSF activates neutrophil elastase, which cleaves membrane-bound SDF-1α from endothelial or stromal cells of bone marrow and provokes an efflux of stem cells to loci of high SDF-1α concentration. This is consistent with in vitro data showing transmigration capacity in response to an SDF-1α gradient.[21]

Injection of human SDF-1α into the spleen and bone marrow of immunodeficient mice with severe combined immunodeficient syndrome has led to rapid homing of transplanted human stem cells into the spleen and bone marrow. SDF-1α expression is increased upon tissue injury, not only in the bone marrow microenvironment, but also in several other organs, including the heart, liver, and brain, to recruit HPCs participating in tissue regeneration.[5,20] Thus, SDF-1α appears to play a crucial role in the homing of HPCs into injured tissue to promote regeneration.

SDF-1α is expressed in various tissues of adult rats. Upon nerve crush injury, there is a transient increase in the level of SDF-1 mRNA. It reaches a maximum of 175% at 2 days after injury and declines to control level at 7 days after injury.[11] However, the involvement of HPCs in nerve regeneration triggered by SDF-1 expressed in injured nerve has not been determined.

In this study, we postulate that SDF-1α in injured nerve regulates the integration of $CD34^+$ cells into the nerve tissue to promote its regeneration. We investigate the potency of SDF-1α expressed in injured nerve to attract $CD34^+$ cells to injured nerve tissue as well as strengthening or weakening these effects with local application of SDF-1α recombinant protein or SDF-1α antibody. Furthermore, the interaction between increased deposits of $CD34^+$ cells and the expression of angiogenesis factors as well as regeneration markers were also evaluated.

Methods

Animals and Group Assignments

One hundred forty-seven Sprague-Dawley rats (weight 250–300 g) were used in this study. The animals were assigned to 3 groups of 49 rats each. Sciatic nerve crush injury was induced in all animals as described below. The Group 1 rats did not receive any SDF-1α or SDF-1α antibody and served as untreated controls. In the Group 2 and Group 3 animals, the injured nerve was wrapped with woven Surgicel (Johnson & Johnson) impregnated with either SDF-1α recombinant protein (0.1 μg/20 μl; Peprotech; Group 2) or anti–human SDF-1α antibody (2 μg/20 μl; R&D; Group 3). All animals underwent one neurobehavioral assessment session (on metal mesh) per week. Food and water were provided ad libitum. The animals were kept in a temperature-controlled environment at 37°C and were exposed to alternating light and dark periods of 12 hours each. All animals were treated and cared for in accordance with the guidelines recommended by the ethics committee of Taichung Veterans General Hospital.

Crush Injury Model

Anesthesia was induced with 4% isoflurane and maintained with 1%–2% isoflurane. The left sciatic nerve was exposed under the microscope using the gluteal muscle–splitting method. A vessel clamp (B-3, pressure 1.5 g/mm^2, S&T Marketing, Ltd.) was applied 10 mm from the internal obturator canal for 20 minutes. The crush site was marked by placing a 9-0 nylon suture in the epineurium.[33]

Neurobehavioral Studies

Six rats from each group were assessed at intervals of 1 week for the evaluation of sciatic nerve function and quantitative gait analysis.

Analysis of Functional Recovery

A technician who was blinded to group allocation evaluated sciatic nerve function once a week for 4 weeks after the surgery. The evaluation method included ankle

kinematics[51] and SFI assessment.[2] In the sagittal plane analysis, the following formula was used for the mechanical analysis of rat ankle kinematics: θ ankle = θ foot – θ leg (the ankle angle being estimated as the difference between the foot angle and the leg angle, where θ represents angle).

For calculating the SFI, the rat's paw print was measured as follows: 1) distance from the heel to the third toe, the print length (PL); 2) distance from the first to the fifth toes, the toe spread (TS); and 3) distance from the second to the fourth toes, the intermediary toe spread (ITS). All 3 measurements were taken from the experimental (E) and normal (N) sides. The SFI was calculated according to the following equation: SFI = 38.3([EPL – NPL]/NPL) + 109.5([ETS – NTS]/NTS) + 13.3([EITS – NITS]/NITS) – 8.8. The SFI is close to 0 in animals with normal nerve function, whereas an SFI of –100 represents total dysfunction.

Automated Quantitative Gait Analysis

Computerized gait analysis was performed as previously described.[24] Briefly, animals had to cross a 100-cm-long glass floor plate, confined by Plexiglas walls spaced 8 cm apart in a darkened room. Light from an otherwise completely encased white fluorescent tube entered one of the long edges of the glass floor. The light tube was placed 2 cm from the glass plate so that light entering the 5-mm-thick glass plate was completely internally reflected. Only at those points where a paw touched the glass plate did light exit the plate through scattering, thereby illuminating the points of contact of the paws. During crossings, the walkway was monitored from below by a Pulnix TM-62EX camera (Pulnix, Inc.) equipped with a wide-angle objective (8.5 mm, Cosmicar). The camera registered the paw-floor contact in pixels of 1.23 mm^2, with the intensity (gray value) of the signal depending on the applied pressure. Higher paw pressure resulted in a larger total area of skin-floor contact and a more intense scatter—that is, brighter pixels. The signal was digitized by a pcImage-SRGB frame grabber board (Matrix Vision GmbH) and subsequently acquired, compressed, and stored by CatWalk software (Noldus Information Technology) for further analysis. All areas containing pixels brighter than a preset analysis threshold were stored. Using the analysis component of the CatWalk program, these areas were assigned to one of the paws and data were exported to a spreadsheet. Quantitative analysis of these data included the following parameters.

1. Step sequence distribution. Six different walking patterns or normal step sequence patterns that fall into 3 different categories can be discerned in rats, depending on the sequential placement of the 4 paws.

2. Regularity index. This parameter is a measure of interlimb coordination. Interlimb coordination is considered normal when, during uninterrupted locomotion, only normal step sequences are used. The RI rates the degree of interlimb coordination as a percentage of complete coordination by the following equation: RI = (NSSP × 4/PP) × 100%, where NSSP represents the number of normal step sequence patterns and PP indicates the total number of paw placements. Consequently, extra paw placements and irregular walking on 3 paws will result in a decrease of RI.

3. Print area. This parameter was defined as the total floor area in pixels contacted by the paw during stance phase. Possible reasons for increase in the hind limb print area are paralysis of the lower limb leading to a deficiency in plantar stepping or paw/toe dragging during part of the step cycle. A decrease in this parameter can be indicative of mechanical allodynia.

4. Base of support. The distance in millimeters between the 2 hind paws is defined as the base of support. This distance is measured perpendicular to the direction of walking.

5. Duration of stance and swing phase. Since duration of stance or swing phase depends on the animal's walking speed and degree of dysfunction, these parameters are transformed to a fraction of total step duration according to the following formula: fraction stance or swing phase = [time in stance or swing phase/(time single step)] × 100%. Time of stance phase or swing phase and total step are expressed in seconds.

6. Hind paw pressure. This is the mean intensity of the contact area of the hind paw at the moment of maximum paw-floor contact. This parameter is expressed in arbitrary units (intensity arbitrary units).

Electrophysiological Study

Four weeks after the crush injury, all 18 animals used for the behavioral studies were anesthetized as for the original procedure and their bilateral sciatic nerves were exposed. Electrical stimulation was applied to the proximal side of the injured site. The evoked CMAP amplitudes and conduction latency were recorded in the gastrocnemius with an active monopolar needle electrode 10 mm below the tibial tuberosity and with a reference needle 20 mm from the active electrode. The stimulation intensity was 5 mA and the filtration range was 20–2000 Hz. A similar assessment was performed on the noninjured side. The CMAP data and conduction latency were converted to a ratio (the injured side divided by the normal side) to adjust for the effect of anesthesia.[33]

Histological Examination

After neurobehavioral and electrophysiological testing, 4 weeks after crush injury, the 18 rats (6 per group) were reanesthetized with pentobarbital (30 mg/kg) and then euthanized by means of transcardial perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The left sciatic nerve was harvested and the nerve tissue was fixed on a plastic plate using stay suturing to keep the nerve straight.[24] The bilateral gastrocnemius muscles were dissected from the bones and sent for measurement of muscle weight. The nerve was embedded in paraffin and cut longitudinally (parallel to the long axis of the axon) into sections 8-μm thick, which were then stained with H & E for the measurement of vacuole number or subjected to immunohistochemical staining with S100 protein for determination of myelination. The numbers of vacuoles and density of S100-positive cells were determined as previously described.[32]

Western Blot Analysis

Three rats from each group were used for Western blot analysis at 1, 3, 7, 10, 14, 21, and 28 days after crush injury (a total of 63 rats). The animals were euthanized by inhalation of CO_2 and then a 10-mm specimen of nerve tissue (extending 5 mm on each side of the suture marker) was harvested and proteins were extracted. Proteins (50 μg) were resolved by SDS-polyacrylamide gel electrophoresis and transferred onto a blotting membrane. After blocking with nonfat milk, the membranes were incubated with antibodies against CD34, CD68, SDF-1, CXCR-4, VEGF, and GAPDH (Santa Cruz Biotechnology, Inc.) overnight at 4°C. The membranes were next incubated with horseradish peroxidase–conjugated secondary antibody and developed using enhanced chemoluminescence Western blotting reagents. The intensity of protein bands was determined by means of a computer image analysis system (IS 1000, Alpha Innotech Corporation).

Transwell Migration Assays

One week after crush injury, 1 animal from each group was euthanized by means of CO_2 inhalation, and bone marrow was collected from the bilateral long bones as follows. The bones were dissected, and the proximal and distal ends were removed to reveal the marrow cavity, which was aspirated with 10 ml of 0.01 M phosphate-buffered saline through a 21-gauge needle. The aspirate was filtered through a 70-μm filter (Falcon) to remove bone fragments for migration analysis.

The bone marrow aspirates were suspended in RPMI-1640 medium and seeded into Millicell hanging cell-culture inserts (5-μm pore size, 10^6 cells per insert; Millipore Corporation). Migration-inducing medium was placed in the chambers of the 24-well plates (Corning Costar Corporation), which contained different concentrations of recombinant SDF-1α (5, 10, and 20 μmol/L) or 0.1% of DMSO as the solvent control. Then, the plates were placed into a humidified incubator. After 12 hours, assays were stopped by removal of the insert, and migrated cells were collected from the 24-well plates. The total number of cells in each well was counted and the contents of each well were centrifuged separately at 400 G for 5 minutes at 25°C to obtain 1 pellet per well. The cell pellet was suspended in 2-ml 0.01 M phosphate-buffered saline for centrifugation using a Cytospin centrifuge (Thermo Scientific). Slides and filters were placed into appropriate slots in the centrifuge with cardboard filters facing the center, and 100 μl of each sample was placed in the appropriate wells of the centrifuge. The lid was placed on the centrifuge and the samples were spun at 600 rpm for 3 minutes. Each slide was examined under the microscope to be sure that the cells annealed properly. Slides were dried in a desiccation chamber overnight. The slides were subjected to immunohistochemical staining with antibodies against CD34, CD68, CD45, and DAPI. Five areas (of 0.01 mm^2 each) were randomly selected for analysis.

Immunohistochemical Analysis of Injured Nerve Tissue

An additional 3 animals from each group were euthanized (with transcardial perfusion following intraperitoneal injection of pentobarbital) at 1, 3, 7, 10, 14, 21, and 28 days after crush injury and used for immunohistochemical analysis. Serial 8-μm-thick sections of sciatic nerve were cut on a cryostat and mounted on SuperFrost Plus slides (Menzel-Gläser) and were subjected to immunohistochemical analysis using antibodies against CD68, CD34, CD45 (Chemicon, 1:200 dilution), CD8, CD3 (Serotec, 1:200 dilution), CD19 (Thermo Scientific, 1:200 dilution), neutrophil (Abcam, 1:200 dilution), and neurofilament (Chemicon, 1:300 dilution), for detection of inflammatory cells and nerve fibers. The immunoreactive signals were observed using goat anti–mouse IgG (FITC, Jackson, 1:200 dilution), anti–mouse IgG (Rhodamine, Jackson, 1:200 dilution), or 3,3′-diaminobenzidine. Of 100 squares with a surface area of 0.01 mm^2 each, 20 squares were randomly selected in an ocular grid and used for cell counts. For the determination of neurofilament, 6 nerves in each group were cut lengthwise (parallel to the long axis of the axon) into 8-μm-thick sections and stained with each antibody. The region of maximum diameter of the resected nerve tissue with crush mark was chosen to be examined. Areas of immunoreactivity (0.2 mm^2) appeared dense against the background and were measured with a computer image analysis system (IS 1000, Alpha Innotech).

Statistical Analysis

Data were expressed as means ± SEs. The statistical significance of differences between groups was determined by 1-way ANOVA followed by the Dunnett test. For the SFI, ankle angle, and CatWalk analyses, the results were analyzed by repeated-measures ANOVA followed by the Bonferroni multiple comparison method. A p value < 0.05 was considered significant. Data pertaining to muscle weight, CMAP, and conduction latency are presented as ratios (injured-site value divided by normal-site value).

Results

Effect of SDF-1α on Migration of $CD34^+$ Cells In Vitro

SDF-1α expression is increased upon nerve injury, but the intensity of HPC recruitment to injured nerve by SDF-1α has not previously been elucidated. To mimic stimulation of HPC release by expression of SDF-1α in crushed nerve tissue, rat bone marrow cells were harvested and seeded in Millicell hanging cell-culture inserts. Bone marrow cell recruitment was evaluated using various concentrations of SDF-1α. A high proportion of migratory cells (DAPI-positive cells) were $CD34^+$ cells, with a range from 95% ± 2.1% (SDF-1α 5 μg/ml) to 97% ± 1.7% (SDF-1α 20 μg/ml). There were very few immune cells expressing CD68 involved in the migration assay. The density of $CD34^+$ cells was 37 ± 4.1, 155 ± 4.3, and 264 ± 17.9 cells per 0.01 mm^2, respectively, at 5, 10, and 20 μg/ml concentrations of SDF-1α ($p < 0.001$). A significant difference in $CD34^+$ cell density was found between SDF-1α 5 and 10 μg/ml ($p < 0.001$), SDF-1α 5 and 20 μg/ml ($p < 0.001$), and SDF-1α 10 and 20 μg/ml ($p < 0.01$) (Fig. 1). This effect suggests that SDF-1α exerted a homing effect on HPCs,

especially CD34+ cells, the density of which was highly correlated with the concentration of SDF-1α.

Expression of SDF-1α and Associated Proteins

The expression of SDF-1α is increased upon tissue injury not only in the bone marrow microenvironment, but also in several other organs such as heart, liver, and brain, to recruit HPCs for tissue regeneration. However, the recruitment of bone marrow progenitor cells to injured nerve and their regulation by SDF-1α are not fully understood. In the present study, expression of SDF-1α started to increase at 3 days after crush injury (19.3 ± 3.8 intensity arbitrary units) and reached a maximum at 14 days (41.5 ± 3.8 intensity arbitrary units, $p < 0.001$). This trend reflected a similar increase in the expression of the receptor CXCR-4 (measured by Western blot analysis). The increased expression of SDF-1α paralleled the significantly increased expression of CD34 (3.7 ± 0.5 intensity arbitrary units at 3 days [$p < 0.001$] and 17.1 ± 1.3 intensity arbitrary units at 14 days [$p < 0.001$]). However, the expression of SDF-1α was not correlated with that of CD68, which was compatible with the results of the transwell migration study described above (Fig. 2). This indicates that SDF-1α expression upon nerve injury recruits HPCs, especially CD34+ cells, into the injured nerve, and therefore plays a role in nerve regeneration.

Effect of Treatment With SDF-1α Recombinant Protein and SDF-1α Neutralizing Antibodies on SDF-1α Expression

The increased expression of SDF-1α was abolished by the administration of SDF-1α neutralizing antibodies (19.8 ± 2.2 intensity arbitrary units) at Day 14 ($p < 0.001$), whereas the effect was augmented by application of SDF-1α recombinant protein (Fig. 3). In untreated injured animals and in those treated with local application of SDF-1α, the expression of SDF-1α at Day 14 paralleled the significant increase in CD34 expression (22.5 ± 1.9 [$p < 0.01$] and 29.8 ± 1.9 [$p < 0.001$] intensity arbitrary units, respectively) and VEGF expression (15.3 ± 1.5 [$p < 0.001$] and 24.5 ± 3.1 [$p < 0.001$] intensity arbitrary units, respectively). These phenomena were counteracted by the administration of SDF-1α antibodies, which resulted in decreased expression of CD34 (15.3 ± 2.3 intensity arbitrary units) ($p < 0.001$) and VEGF (14.5. ± 2.1 intensity arbitrary units) ($p < 0.001$) (Fig. 4).

Neurobehavior After Crush Injury and Treatment With SDF-1 or Neutralizing Antibodies

In nerve crush injury, SDF-1α has been shown to be involved in recruitment of CD34+ cells, which may parallel the expression of angiogenesis factors to augment the regenerative response.[50] In the current study, animals were administered SDF-1α recombinant protein or neutralized antibodies after crush injury to further verify this hypothesis. The SFI values were significantly better in the untreated injured rats and the rats treated with SDF-1α than in the animals treated with SDF-1α–neutralizing antibodies ($p < 0.01$ and $p < 0.001$, respectively); results in the SDF-1α group were significantly better than those in the control group ($p < 0.01$) (Fig. 5A). The ankle

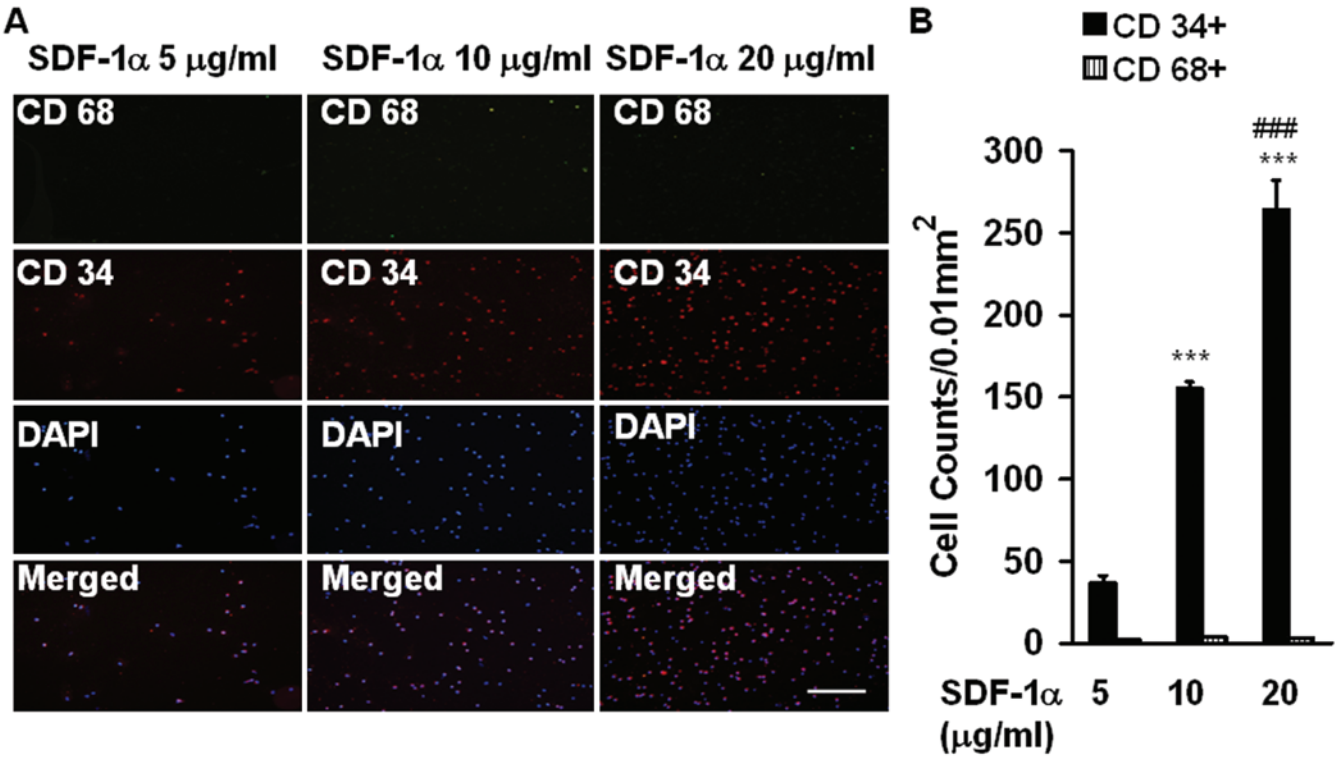

Fig. 1. Migration of HPCs treated with SDF-1α. **A:** Photomicrographs showing transwell migration assay. Original magnification × 200. Bar = 50 μm. **B:** Quantitative analysis of cell numbers of field (HPCs per 0.01 mm^2). ***$p < 0.001$ (relative to SDF-1α 5 μg/ml); ###$p < 0.01$ (relative to SDF-1α 10 μg/ml).

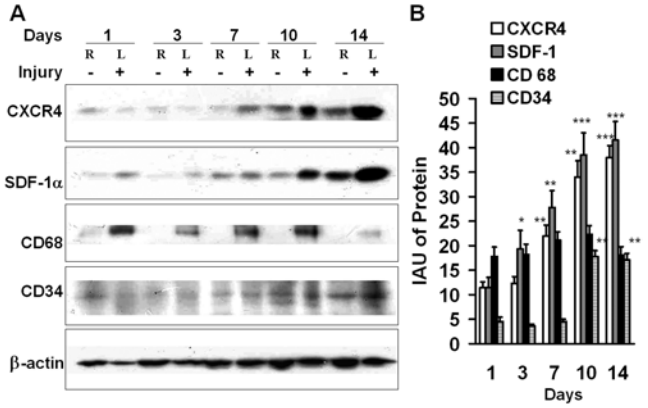

Fig. 2. Expression of SDF-1α and associated protein after sciatic nerve crush injury. **A:** Expression of SDF-1α, CXCR-4, CD68, and CD34 at different time intervals; β-actin was used for calibration. **B:** Results of quantitative analysis at 1, 3, 7, 10, and 14 days after crush injury. IAU = intensity arbitrary units; L = left sciatic nerve (crushed with vessel clamps); R = right sciatic nerve (control). *p < 0.05, **p < 0.01, ***p < 0.001 (vs Day 1).

angle also showed the same trend (Fig. 5B). In the CatWalk analysis, the mean intensity and print area showed a remarkable improvement in the SDF-1α–treated group compared with the untreated injured animals, and the intensity ratios in the antibody-treated rats were even lower than those in the untreated injured controls. With respect to stance duration, the ratio of duration dropped to half of the original value upon injury. Then it demonstrated significant improvement in the SDF-1α group as compared with the untreated controls, whereas the SDF-1α antibody group showed minimal change. With respect to swing duration, the ratio of duration was significantly lower (a sign of better function) in the SDF-1α group than in the untreated group, and it was also significantly lower in the untreated group than in the antibody group (Fig. 6).

Electrophysiological Study and Histological Examination

The parameters of CMAP, conduction latency, and muscle weight also were restored by SDF-1α administration and were further reduced by administration of neutralized antibodies. The mean CMAP amplitude ratio (value from injured side divided by value from uninjured

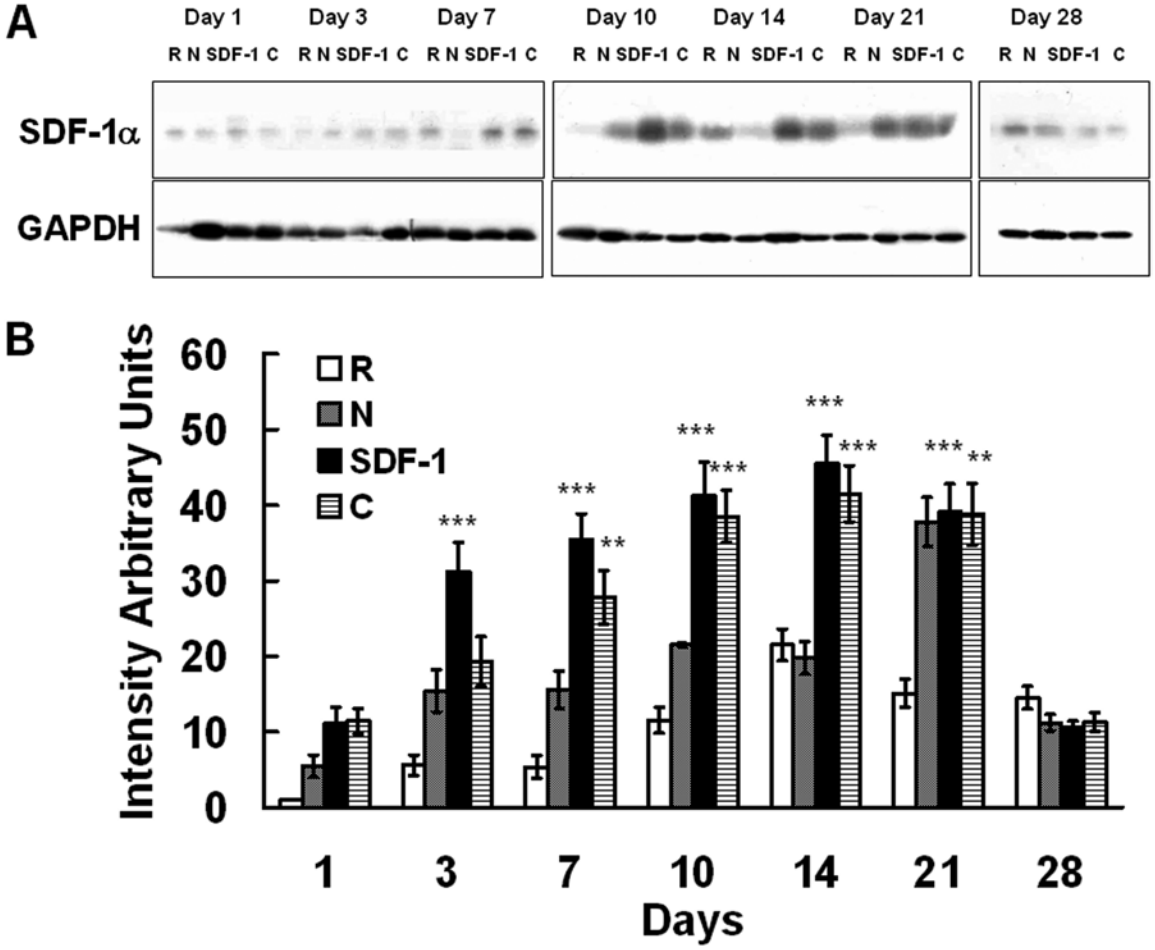

Fig. 3. Expression of SDF-1α profiles treated with either neutralized antibodies or SDF-1α. **A:** Expression of SDF-1α in right and left sciatic nerves treated with neutralized antibodies or SDF-1α recombinant proteins at different time intervals. **B:** Quantitative analysis of SDF-1α based on treatment groups and time intervals. C = crush injury (left sciatic nerve); N = neutralized antibody; R = right sciatic nerve as a control. **p < 0.01; ***p < 0.001 (vs crush injury group [untreated controls]).

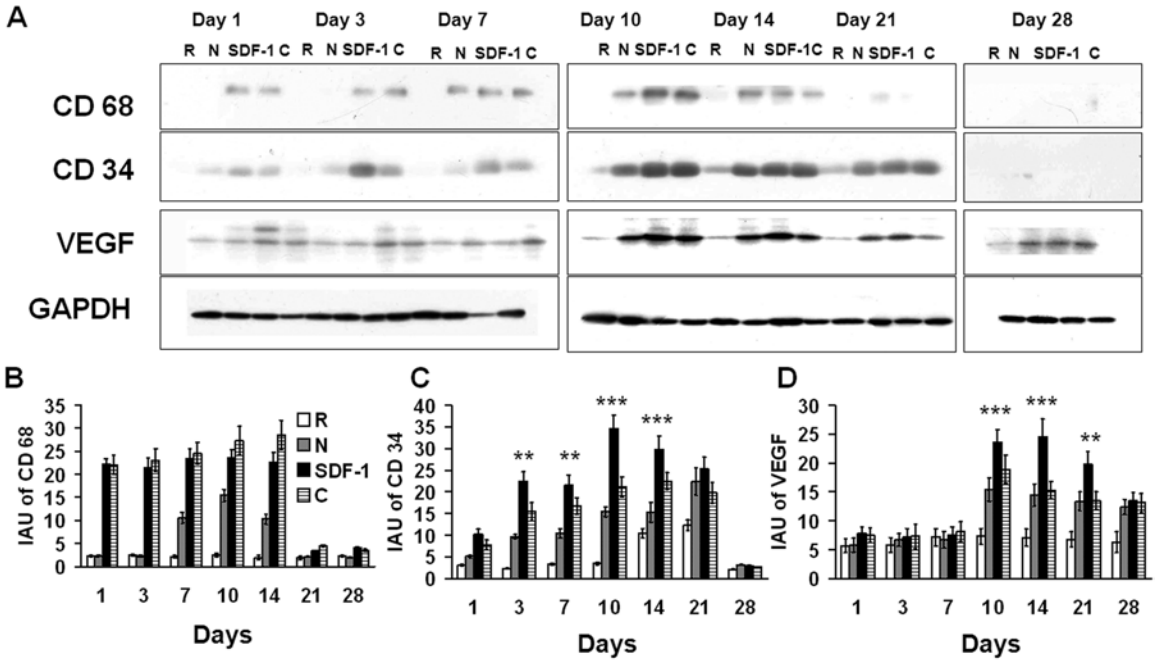

Fig. 4. Expression of angiogenesis factors. **A:** Expression of CD68, CD34, and VEGF based on different treatment modalities. **B–D:** Quantitative analysis of CD68, CD34, and VEGF. **p < 0.01; ***p < 0.001 (vs controls).

side, expressed as a percentage) was 22.1% ± 1.8% in the untreated injured controls, 48.9% ± 3.2% in the SDF-1α–treated animals ($p < 0.05$ vs controls), and 20.9% ± 2.3% in the animals treated with neutralized SDF-1α antibody ($p < 0.05$ vs controls). The latency ratio was 310% ± 20% in the untreated injured controls, 170% ± 10% in the animals treated with SDF-1α ($p < 0.001$ for comparison with controls), and 330% ± 20% in the animals treated with neutralizing antibody ($p < 0.05$ vs controls). The ratio of muscle weight (left/right) was 49.2% ± 1.4% in the controls, 65.7% ± 1.1% in the SDF-1α–treated animals ($p < 0.05$), and 45.8% ± 1.1% in the animals treated with SDF-1α antibody ($p < 0.05$ compared with controls). Hence, administration of SDF-1α promoted nerve regeneration in terms of SFI, muscle weight, CMAP, and conduction latency. The delivery of SDF-1α antibodies contributed to further reduction of this effect (Fig. 5C).

In comparison with the background, the mean relative density per square millimeter of cells expressing S100 was 46.5 ± 2.1 in the untreated controls, 127.1 ± 7.3 in the SDF-1α–treated group ($p < 0.001$), and 39.8 ± 2.1 in the antibody-treated group ($p < 0.05$). The mean vacuole counts were 202 ± 2.1/0.05 mm^2 in the control group, 134.3 ± 7.9/0.05 mm^2 in the SDF-1α group ($p < 0.001$ compared with controls), and 229 ± 8.8/0.05 mm^2 in the SDF-1α antibody group ($p < 0.05$ compared with controls). The quantitative histological findings in terms of expression of myelination fiber and vacuole counts further confirmed these trends (Fig. 7).

Expression of Nerve Regeneration Markers and Associated HPCs in Injured Nerve

Neurofilament is an early marker of nerve regeneration. To determine the expression of neurofilament and its relation with HPC deposition at different time points, it was necessary to obtain serial specimens for analysis of neurofilament, CD34, and CD68 expression. The expression of neurofilament in the crush group increased from 217 ± 13.6 relative density/mm^2 at 7 days and reached 413.2 ± 15.8 relative density/mm^2 at 21 days. The administration of SDF-1α resulted in a significant increase in neurofilament expression (mean relative density per square millimeter: 418.1 ± 15.5 at 7 days and 1017.3 ± 47.8 at 21 days [$p < 0.001$ vs controls for both measurements]). Neurofilament expression was reduced by administration of SDF-1α antibody (mean relative density per square millimeter: 142.5 ± 11.2 at 7 days and 220.1 ± 10.9 at 21 days [$p < 0.05$ vs controls for both]). In the untreated injured controls, the mean number of CD34$^+$ cells per 0.05 mm^2 increased from 8.7 ± 0.9 at 7 days to 16.8 ± 1.1 at 21 days. The administration of SDF-1α resulted in increased CD34 expression, with the mean number of CD34$^+$ cells per 0.05 mm^2 being 14.5 ± 0.7 at 7 days ($p < 0.01$ compared with controls) and 34.5 ± 2.1 at 21 days ($p < 0.001$ compared with controls). This effect was attenuated by administration of neutralizing antibodies, which resulted in mean values of 3.5 ± 0.6 cells/0.05 mm^2 at 7 days ($p < 0.05$ compared with controls) and 3 ± 0.4 cells/0.05 mm^2 at 21 days ($p < 0.05$ compared with controls). In contrast, the mean number of CD68$^+$ cells in specimens from the untreated controls remained relatively high and quite stable from 7 to 14 days after injury (34.2 ± 1.2 cells/0.05 mm^2 at 7 days and 35.5 ± 1.6 cells/0.05 mm^2 at 14 days) and was markedly decreased at 21 days (6.5 ± 0.7 cells/0.05 mm^2 at 21 days). The administration of SDF-1α did not have an effect on CD68 expression (31.5 ± 1.2 cells/0.05 mm^2 at 7 days, 33 ± 1.5

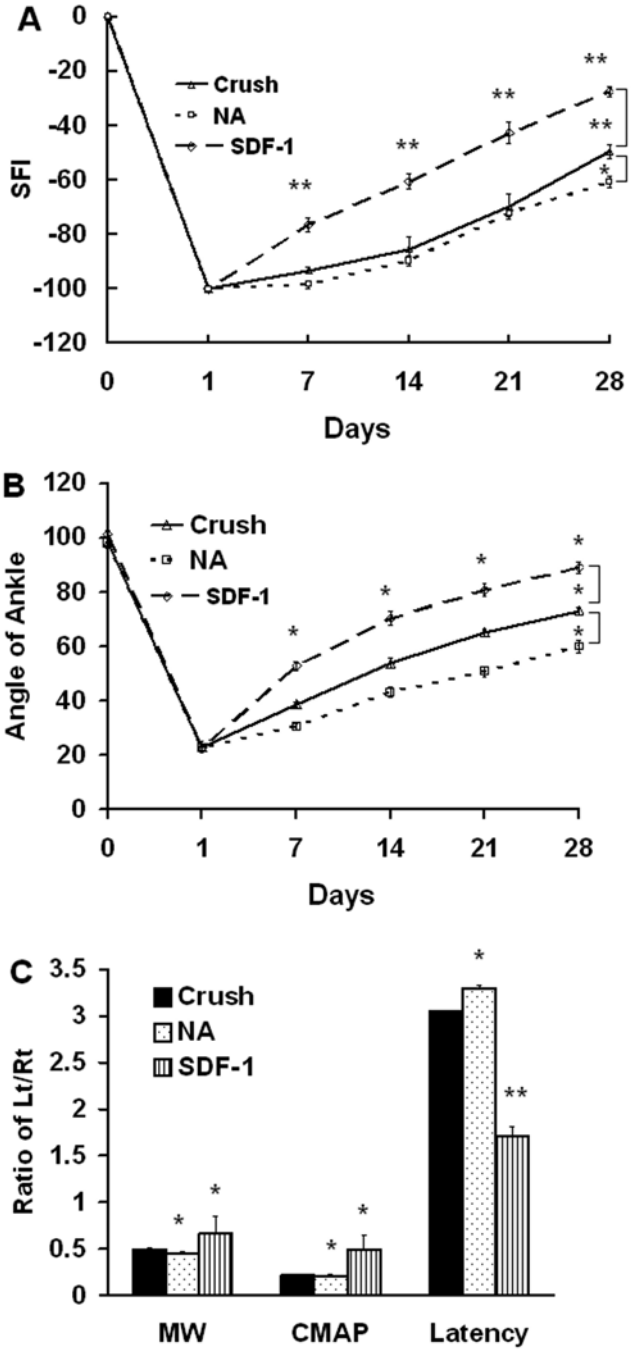

Fig. 5. Results of neurobehavioral and electrophysiological studies in rats with untreated crush injury (crush, *solid line*), crush injury treated with neutralized SDF-1α antibody (NA, *small-dash line*), and crush injury treated with SDF-1α recombinant protein (SDF-1, *large-dash line*). **A and B:** Mean SFI values and ankle angles (in degrees) at different time points for the 3 groups. **C:** Quantitative analysis of the ratios of muscle weight (MW), CMAP, and conduction latency in left (injured) and right (control) legs. Mean values from 6 animals per group. Lt/Rt = left/right. *p < 0.05; **p < 0.01 (vs controls).

cells/0.05 mm^2 at 14 days, and 6 ± 0.4 cells/0.05 mm^2 at 21 days). Similarly, in the animals treated with SDF-1α antibodies, the count was 32 ± 1.1 cells/0.05 mm^2 at 7 days and remained stable (31.2 ± 1.38 cells/0.05 mm^2) at 14 days, decreasing to 7.3 ± 0.9 cells/0.05 mm^2 at 21 days (Fig. 8). Hence, spontaneous regeneration of nerve occurred with progressively increased expression of neurofilament, and this effect was enhanced by SDF-1α administration and eliminated by SDF-1α antibodies. There was a significant positive correlation between neurofilament expression and the numbers of CD34$^+$ cells, but not between neurofilament expression and numbers of CD68$^+$ cells. This further confirmed that SDF-1α expression played a crucial role in the recruitment of CD34$^+$ cells involved in nerve regeneration.

Discussion

Increased expression of SDF-1α in injured nerve occurred upon injury, and this result paralleled the significant recruitment of CD34$^+$ cells to the site of injury. Increased amounts of CD34$^+$ cells in injured nerve correlated with expression of angiogenesis factors. These phenomena were increased by the administration of SDF-1α recombinant protein and partially prevented by administration of neutralizing antibodies. The results of neurobehavioral and electrophysiological evaluations, the degree of myelination of the injured nerve, and the expression of regeneration markers were also consistent with the expression profile of SDF-1α and CD34$^+$ cells. Hence, the expression of SDF-1α appears to exert a homing effect on CD34$^+$ cells by recruiting them in the process of nerve regeneration.

Integration of HPCs into injured tissue has been shown to limit cell loss and promote regeneration in conditions such as brain ischemia, heart ischemia, spinal cord injury, and peripheral nerve injury.[6,19,34,49] Administration of G-CSF activates neutrophil elastase, which cleaves the membrane-bound SDF-1 of endothelial or stromal cells of bone marrow and provokes an efflux of stem cells to loci of high SDF-1α concentration.[21] Furthermore, injection of human SDF-1α into spleen and bone marrow of immunodeficient mice with severe combined immunodeficiency syndrome led to rapid homing of transplanted human stem cells into the spleen and bone marrow.[5,20] In the present study, SDF-1α expression was induced in injured nerve with a peak from 7 to 14 days, and the progressively increased expression of SDF-1α was in line with recruitment of significant amounts of CD34$^+$ cells. This phenomenon was enhanced by the local injection of SDF-1α recombinant protein and partially prevented by SDF-1α neutralized antibody. Hence, SDF-1α expression in injured nerve was highly correlated with the integration of CD34$^+$ cells and paralleled the recovery of neurological function.

SDF-1α has been demonstrated to upregulate the expression of VEGF through the induction of extracellular signal-regulated kinase 1/2 signaling and to induce proliferation of human umbilical vein endothelial cells.[30] Furthermore, the interaction of SDF-1α and CXCR-4 plays a crucial role in ECM-dependent endothelial tube formation as well as in the regulation of endothelial cell morphogenesis and angiogenesis.[41] On the other hand, the parallel recruitment of CD34$^+$ cells in the injured nerve

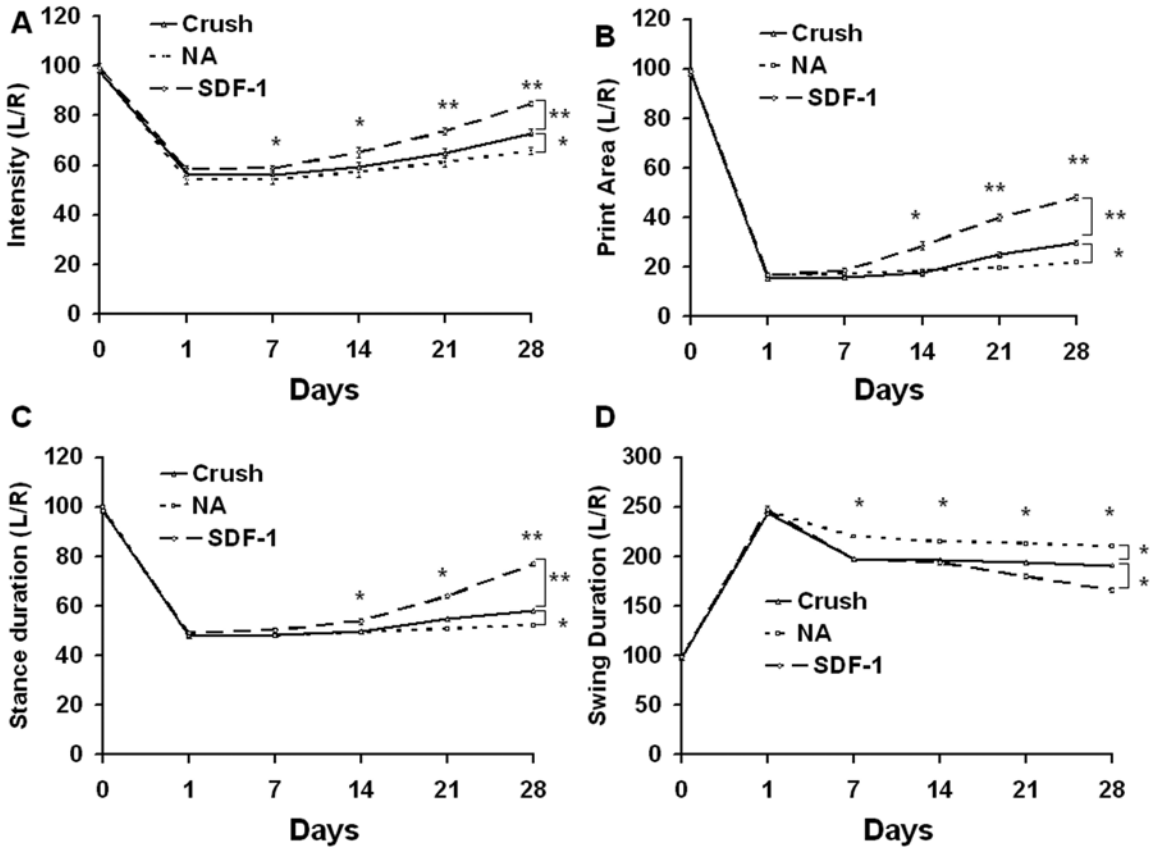

Fig. 6. CatWalk analysis based on different treatment modalities and time points. **A:** Ratio of mean intensity values (left [injured side]/right [uninjured]). **B:** Paw print area ratio (left/right). **C:** Stance duration ratio (left/right). **D:** Swing duration ratio (left/right). Values are group means; 6 rats per group. L/R = left/right. *$p < 0.05$, **$p < 0.01$ (vs controls).

has been shown to be responsible for elevation of VEGF.[34] In this study, expression of VEGF was in accordance with SDF-1α and CD34 expression profiles, either augmented by SDF-1α recombinant proteins or reduced by neutralized antibody. Thus, elevation of SDF-1α not only recruited $CD34^+$ cells but also exerted angiogenesis effects on $CD34^+$ cells. This effect was responsible for the significant expression of VEGF in the injured nerve, which is an essential requirement for nerve regeneration.

$CD34^+$ cells represent a heterogeneous population at many different stages of functional differentiation, from pluripotent stem cells to lineage-restricted progenitor cells. Most $CD34^+$ cells are progenitors for myeloid and lymphoid lineages, but a subpopulation, defined by the expression of c-kit receptors and lack of expression of CD38 and hematopoietic lineage markers, is considered to be composed of pluripotent HPCs that can give rise to all hematopoietic lineages.[13] Recently, $CD34^+$ cells have been widely applied to various categories of regenerative medicine. In the immunodeficient rat model of acute myocardial infarction, transplanted human $CD34^+$ cells incorporated into the site of myocardial neovascularization, differentiated into mature endothelial cells, augmented capillary density, inhibited myocardial fibrosis, and preserved left ventricular function.[16–18] Systemic administration of human cord blood–derived $CD34^+$ cells to immunocompromised mice 48 hours after induction of stroke induces neovascularization in the ischemic zone and provides a favorable environment for neuronal regeneration.[47] Furthermore, transplantation of $CD34^+$ cells promotes revascularization and augmented wound healing in full-thickness skin wounds of diabetic mice.[45] $CD34^+$ cells also augmented bone fracture and ligament healing with involvement of angiogenesis.[28,48] Significantly, $CD34^+$ cells also have the potential to differentiate into neurons and become part of the brain or spinal cord.[44,53,54] In our previous report, we also found that $CD34^+$ cells were integrated into injured nerve without differentiation into Schwann or neuronal cells.[50] The expression of angiogenesis factors did not colocalize with $CD34^+$ cells; this failure to colocalize $CD34^+$ cells with angiogenesis factors indicates the paracrine effects of the $CD34^+$ cells.[50] In this study, the accumulation of $CD34^+$ cells was in line with the expression of VEGF without the potentiation of lineage differentiation, and this result supports the findings reported in our previous study.

During nerve regeneration, myelination of nerve fiber was used as a marker of the extent of nerve regeneration.[52] Neurofilament expression provided early evidence of nerve regeneration potential.[31] The amount of S100 immunoreactivity in myelinated fibers appeared to be directly correlated with thickness of the myelin sheath formed by Schwann cells.[27] Further, vacuole formation and vascular staining reflected the extent of nerve regeneration.[32]

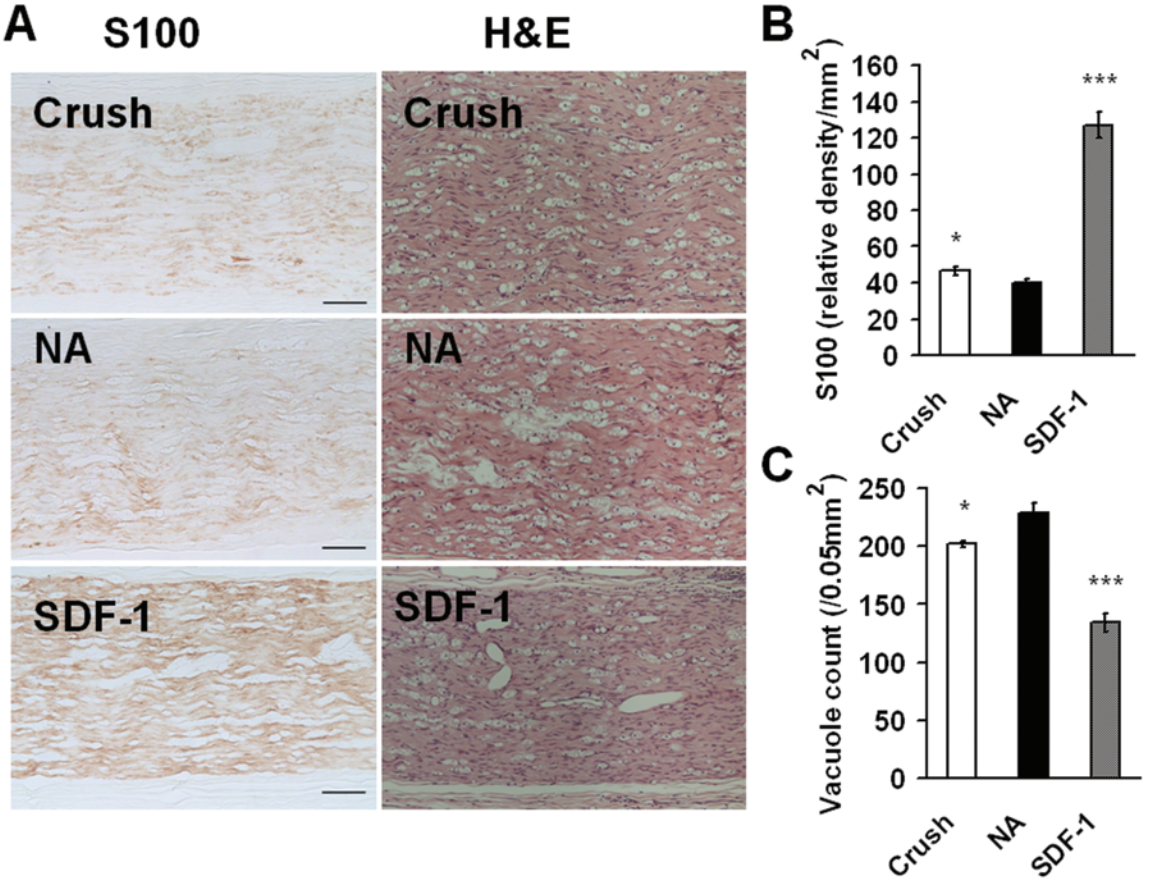

Fig. 7. Histological findings in sciatic nerve injury. Left sciatic nerves were crushed and treated with either neutralized antibodies or SDF-1α recombinant protein or left untreated. The nerves were retrieved for analysis 28 days after injury. **A:** Photomicrographs showing expression of S100 and relative numbers of vacuoles in the 3 treatment groups. **B and C:** Quantitative analysis of S100 expression **(B)** and vacuole counts **(C).** *p < 0.05, **p < 0.01, ***p < 0.001 (vs controls).

Hence, in this study, we used neurofilament as an early marker as well as the expression of immunoreactivity of S100 and vacuole counts as late markers to evaluate the extent of nerve regeneration. The significant expression of SDF-1α, $CD34^+$ cells, and VEGF paralleled the elevation of nerve regeneration markers, which further confirmed the hypothesis that involvement of $CD34^+$ cells in nerve regeneration is influenced by SDF-1α expression.

Spontaneous recovery of motor function in a sciatic nerve crush injury model had been discussed several decades ago, and it has recently become a topic of interest again.[7,52] In our previous studies, we found that nerve regeneration in a crush injury experimental model did not reach full recovery and left serious neurological deficits 4 weeks postinjury.[32,33] There were several possibile factors contributing to this reported spontaneous recovery of motor function, including secretion of neurotrophic and angiogenesis factors.[4,10,14,25,40,42,46] In this study, the significant expression of SDF-1α was correlated with increased recruitment of $CD34^+$ cells and was highly correlated with expression of VEGF. This effect was further augmented by administration of SDF-1α and was reduced by administration of SDF-1α antibody. This indicates that SDF-1α exerts its influence by attracting hematopoietic progenitors to recruit them in peripheral nerve regeneration.

Conclusions

The elevation of SDF-1α at different time points after crush injury paralleled the significant recruitment of $CD34^+$ cells and was highly correlated with expression of VEGF. These effects were in line with improvements in neurobehavior and increased levels of regeneration markers. Also, the effects were further augmented by the administration of SDF-1α recombinant protein and prevented by administration of the neutralizing antibody. Hence, SDF-1α triggered recruitment of HPCs involved in nerve regeneration. This strategy may be considered as a treatment alternative in peripheral nerve disease.

Disclosure

This study was supported by grants from Taichung Veterans General Hospital, Providence University (TCVGH-NCHU987611), and the National Science Council (NSC-99-2314-B-075A-005-MY2), Taiwan, R.O.C. The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author contributions to the study and manuscript preparation include the following. Conception and design: Pan, Cheng, CJ Chen, Chiu. Acquisition of data: Pan, Sheu. Analysis and interpretation of data: Pan, Sheu, Su, YJ Chen. Drafting the article: Pan, Sheu. Critically revising the article: Cheng, Sheehan. Approved the final

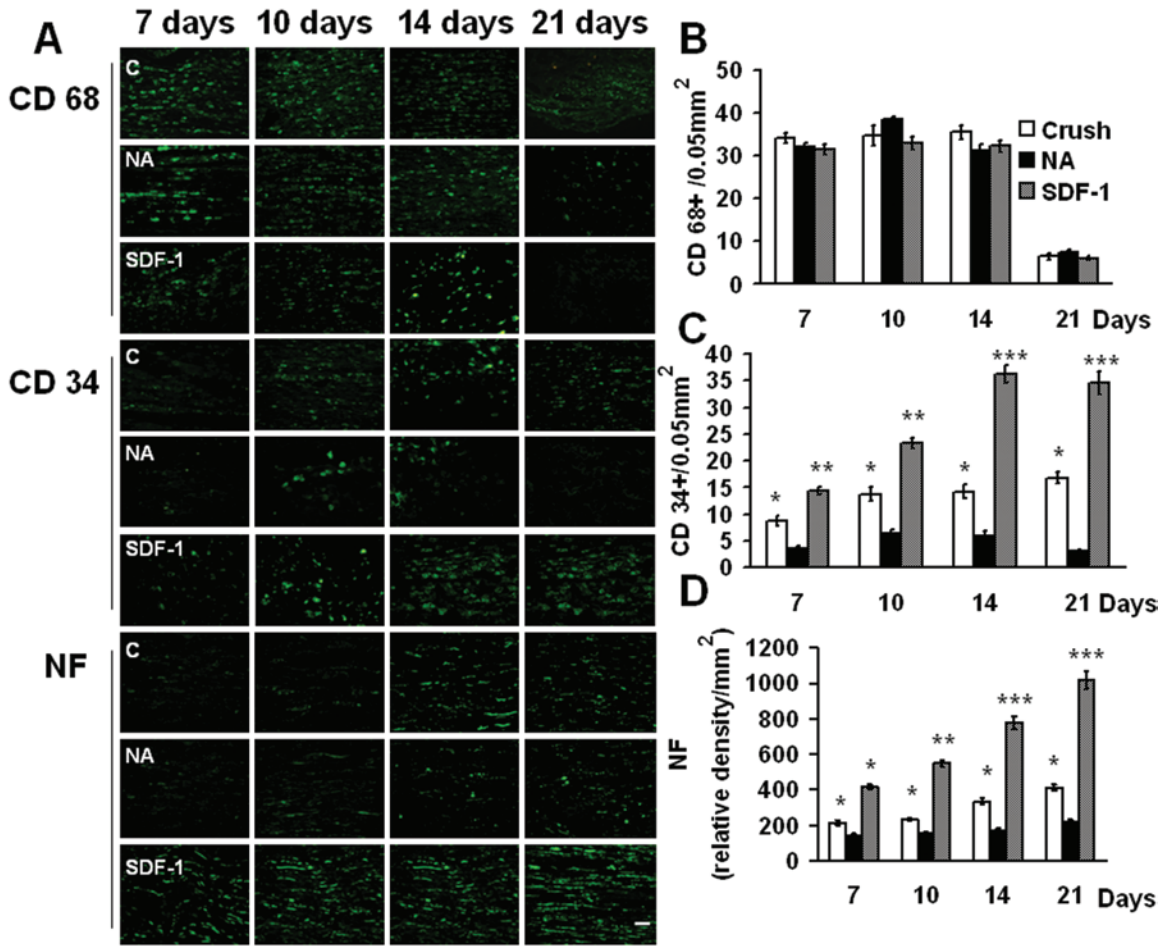

Fig. 8. Expression of CD68, CD34, and neurofilament (NF) based on different treatment modalities and time intervals. **A:** Photomicrographs showing CD68, CD34, and neurofilament expression at different time intervals. Bar = 50 μm. **B–D:** Results of quantitative analysis of CD68, CD34, and neurofilament expression. *p < 0.05, **p < 0.01, ***p < 0.001 (vs controls).

version of the paper on behalf of all authors: Pan. Statistical analysis: YJ Chen. Administrative/technical/material support: CJ Chen, Chiang, Chiu. Study supervision: Pan.

Acknowledgments

The authors thank Mrs. Shu-Zhen Lai, Mrs. Lin-Lan Yang, and Mrs. Mu-Jung Liu for helping to prepare the manuscript, and the Biostatistics Task Force of Taichung Veterans General Hospital for their assistance with the statistical analysis.

References

1. Abkowitz JL, Robinson AE, Kale S, Long MW, Chen J: Mobilization of hematopoietic stem cells during homeostasis and after cytokine exposure. **Blood 102:**1249–1253, 2003
2. Bain JR, Mackinnon SE, Hunter DA: Functional evaluation of complete sciatic, peroneal, and posterior tibial nerve lesions in the rat. **Plast Reconstr Surg 83:**129–138, 1989
3. Chen CJ, Ou YC, Liao SL, Chen WY, Chen SY, Wu CW, et al: Transplantation of bone marrow stromal cells for peripheral nerve repair. **Exp Neurol 204:**443–453, 2007
4. Chen ZY, Chai YF, Cao L, Lu CL, He C: Glial cell line-derived neurotrophic factor enhances axonal regeneration following sciatic nerve transection in adult rats. **Brain Res 902:** 272–276, 2001
5. Cheng FH, Tai MH, Sheu ML, Chen CJ, Yang DY, Su HL, et al: Enhancement of regeneration with glia cell line–derived neurotrophic factor–transduced human amniotic fluid mesenchymal stem cells after sciatic nerve crush injury. Laboratory investigation. **J Neurosurg 112:**868–879, 2010
6. Cheng Z, Liu X, Ou L, Zhou X, Liu Y, Jia X, et al: Mobilization of mesenchymal stem cells by granulocyte colony-stimulating factor in rats with acute myocardial infarction. **Cardiovasc Drugs Ther 22:**363–371, 2008
7. de Medinaceli L, Freed WJ, Wyatt RJ: An index of the functional condition of rat sciatic nerve based on measurements made from walking tracks. **Exp Neurol 77:**634–643, 1982
8. Dezawa M, Takahashi I, Esaki M, Takano M, Sawada H: Sciatic nerve regeneration in rats induced by transplantation of in vitro differentiated bone-marrow stromal cells. **Eur J Neurosci 14:**1771–1776, 2001
9. Fernandes M, Valente SG, Fernandes MJ, Félix EP, Mazzacoratti MdaG, Scerni DA, et al: Bone marrow cells are able to increase vessels number during repair of sciatic nerve lesion. **J Neurosci Methods 170:**16–24, 2008
10. Frostick SP, Yin Q, Kemp GJ: Schwann cells, neurotrophic factors, and peripheral nerve regeneration. **Microsurgery 18:** 397–405, 1998
11. Gleichmann M, Gillen C, Czardybon M, Bosse F, Greiner-Petter R, Auer J, et al: Cloning and characterization of SDF-1gamma, a novel SDF-1 chemokine transcript with developmentally regulated expression in the nervous system. **Eur J Neurosci 12:**1857–1866, 2000

12. Hasegawa T, Kosaki A, Shimizu K, Matsubara H, Mori Y, Masaki H, et al: Amelioration of diabetic peripheral neuropathy by implantation of hematopoietic mononuclear cells in streptozotocin-induced diabetic rats. **Exp Neurol 199:**274–280, 2006
13. Ikehara S: Pluripotent hemopoietic stem cells in mice and humans. **Proc Soc Exp Biol Med 223:**149–155, 2000
14. Islamov RR, Chintalgattu V, Pak ES, Katwa LC, Murashov AK: Induction of VEGF and its Flt-1 receptor after sciatic nerve crush injury. **Neuroreport 15:**2117–2121, 2004
15. Jeong JO, Kim MO, Kim H, Lee MY, Kim SW, Ii M, et al: Dual angiogenic and neurotrophic effects of bone marrow-derived endothelial progenitor cells on diabetic neuropathy. **Circulation 119:**699–708, 2009
16. Kawamoto A, Gwon HC, Iwaguro H, Yamaguchi JI, Uchida S, Masuda H, et al: Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. **Circulation 103:**634–637, 2001
17. Kawamoto A, Tkebuchava T, Yamaguchi J, Nishimura H, Yoon YS, Milliken C, et al: Intramyocardial transplantation of autologous endothelial progenitor cells for therapeutic neovascularization of myocardial ischemia. **Circulation 107:** 461–468, 2003
18. Kocher AA, Schuster MD, Szabolcs MJ, Takuma S, Burkhoff D, Wang J, et al: Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. **Nat Med 7:**430–436, 2001
19. Koda M, Nishio Y, Kamada T, Someya Y, Okawa A, Mori C, et al: Granulocyte colony-stimulating factor (G-CSF) mobilizes bone marrow-derived cells into injured spinal cord and promotes functional recovery after compression-induced spinal cord injury in mice. **Brain Res 1149:**223–231, 2007
20. Kollet O, Shivtiel S, Chen YQ, Suriawinata J, Thung SN, Dabeva MD, et al: HGF, SDF-1, and MMP-9 are involved in stress-induced human CD34+ stem cell recruitment to the liver. **J Clin Invest 112:**160–169, 2003
21. Kollet O, Spiegel A, Peled A, Petit I, Byk T, Hershkoviz R, et al: Rapid and efficient homing of human CD34(+)CD38(-/low) CXCR4(+) stem and progenitor cells to the bone marrow and spleen of NOD/SCID and NOD/SCID/B2m(null) mice. **Blood 97:**3283–3291, 2001
22. Lemoli RM, Catani L, Talarico S, Loggi E, Gramenzi A, Baccarani U, et al: Mobilization of bone marrow-derived hematopoietic and endothelial stem cells after orthotopic liver transplantation and liver resection. **Stem Cells 24:**2817–2825, 2006
23. Lévesque JP, Hendy J, Takamatsu Y, Simmons PJ, Bendall LJ: Disruption of the CXCR4/CXCL12 chemotactic interaction during hematopoietic stem cell mobilization induced by GCSF or cyclophosphamide. **J Clin Invest 111:**187–196, 2003
24. Lin KL, Yang DY, Chu IM, Cheng FC, Chen CJ, Ho SP, et al: DuraSeal as a ligature in the anastomosis of rat sciatic nerve gap injury. **J Surg Res 161:**101–110, 2010
25. Lohof AM, Ip NY, Poo MM: Potentiation of developing neuromuscular synapses by the neurotrophins NT-3 and BDNF. **Nature 363:**350–353, 1993
26. Massa M, Rosti V, Ferrario M, Campanelli R, Ramajoli I, Rosso R, et al: Increased circulating hematopoietic and endothelial progenitor cells in the early phase of acute myocardial infarction. **Blood 105:**199–206, 2005
27. Mata M, Alessi D, Fink DJ: S100 is preferentially distributed in myelin-forming Schwann cells. **J Neurocytol 19:**432–442, 1990
28. Matsumoto T, Kawamoto A, Kuroda R, Ishikawa M, Mifune Y, Iwasaki H, et al: Therapeutic potential of vasculogenesis and osteogenesis promoted by peripheral blood CD34-positive cells for functional bone healing. **Am J Pathol 169:**1440–1457, 2006
29. Murakami T, Fujimoto Y, Yasunaga Y, Ishida O, Tanaka N, Ikuta Y, et al: Transplanted neuronal progenitor cells in a peripheral nerve gap promote nerve repair. **Brain Res 974:**17–24, 2003
30. Neuhaus T, Stier S, Totzke G, Gruenewald E, Fronhoffs S, Sachinidis A, et al: Stromal cell-derived factor 1alpha (SDF-1alpha) induces gene-expression of early growth response-1 (Egr-1) and VEGF in human arterial endothelial cells and enhances VEGF induced cell proliferation. **Cell Prolif 36:**75–86, 2003
31. Omura K, Ohbayashi M, Sano M, Omura T, Hasegawa T, Nagano A: The recovery of blood-nerve barrier in crush nerve injury—a quantitative analysis utilizing immunohistochemistry. **Brain Res 1001:**13–21, 2004
32. Pan HC, Chen CJ, Cheng FC, Ho SP, Liu MJ, Hwang SM, et al: Combination of G-CSF administration and human amniotic fluid mesenchymal stem cell transplantation promotes peripheral nerve regeneration. **Neurochem Res 34:**518–527, 2009
33. Pan HC, Cheng FC, Chen CJ, Lai SZ, Lee CW, Yang DY, et al: Post-injury regeneration in rat sciatic nerve facilitated by neurotrophic factors secreted by amniotic fluid mesenchymal stem cells. **J Clin Neurosci 14:**1089–1098, 2007
34. Pan HC, Wu HT, Cheng FC, Chen CH, Sheu ML, Chen CJ: Potentiation of angiogenesis and regeneration by G-CSF after sciatic nerve crush injury. **Biochem Biophys Res Commun 382:**177–182, 2009
35. Pan HC, Yang DY, Chiu YT, Lai SZ, Wang YC, Chang MH, et al: Enhanced regeneration in injured sciatic nerve by human amniotic mesenchymal stem cell. **J Clin Neurosci 13:**570–575, 2006
36. Perin EC, Dohmann HF, Borojevic R, Silva SA, Sousa AL, Mesquita CT, et al: Transendocardial, autologous bone marrow cell transplantation for severe, chronic ischemic heart failure. **Circulation 107:**2294–2302, 2003
37. Pollock M: Nerve regeneration. **Curr Opin Neurol 8:**354–358, 1995
38. Prockop DJ: Marrow stromal cells as stem cells for nonhematopoietic tissues. **Science 276:**71–74, 1997
39. Ribeiro-Resende VT, Pimentel-Coelho PM, Mesentier-Louro LA, Mendez RM, Mello-Silva JP, Cabral-da-Silva MC, et al: Trophic activity derived from bone marrow mononuclear cells increases peripheral nerve regeneration by acting on both neuronal and glial cell populations. **Neuroscience 159:**540–549, 2009
40. Rush RA, Mayo R, Zettler C: The regulation of nerve growth factor synthesis and delivery to peripheral neurons. **Pharmacol Ther 65:**93–123, 1995
41. Salvucci O, Yao L, Villalba S, Sajewicz A, Pittaluga S, Tosato G: Regulation of endothelial cell branching morphogenesis by endogenous chemokine stromal-derived factor-1. **Blood 99:**2703–2711, 2002
42. Sendtner M, Arakawa Y, Stöckli KA, Kreutzberg GW, Thoenen H: Effect of ciliary neurotrophic factor (CNTF) on motoneuron survival. **J Cell Sci Suppl 15:**103–109, 1991
43. Shen ZL, Lassner F, Becker M, Walter GF, Bader A, Berger A: Viability of cultured nerve grafts: an assessment of proliferation of Schwann cells and fibroblasts. **Microsurgery 19:**356–363, 1999
44. Sigurjonsson OE, Perreault MC, Egeland T, Glover JC: Adult human hematopoietic stem cells produce neurons efficiently in the regenerating chicken embryo spinal cord. **Proc Natl Acad Sci U S A 102:**5227–5232, 2005
45. Sivan-Loukianova E, Awad OA, Stepanovic V, Bickenbach J, Schatteman GC: CD34+ blood cells accelerate vascularization and healing of diabetic mouse skin wounds. **J Vasc Res 40:**368–377, 2003
46. Sondell M, Lundborg G, Kanje M: Vascular endothelial growth factor has neurotrophic activity and stimulates axonal outgrowth, enhancing cell survival and Schwann cell prolif-

eration in the peripheral nervous system. **J Neurosci 19:**5731–5740, 1999
47. Taguchi A, Soma T, Tanaka H, Kanda T, Nishimura H, Yoshikawa H, et al: Administration of CD34+ cells after stroke enhances neurogenesis via angiogenesis in a mouse model. **J Clin Invest 114:**330–338, 2004
48. Tei K, Matsumoto T, Mifune Y, Ishida K, Sasaki K, Shoji T, et al: Administrations of peripheral blood CD34-positive cells contribute to medial collateral ligament healing via vasculogenesis. **Stem Cells 26:**819–830, 2008
49. Toth ZE, Leker RR, Shahar T, Pastorino S, Szalayova I, Asemenew B, et al: The combination of granulocyte colony-stimulating factor and stem cell factor significantly increases the number of bone marrow-derived endothelial cells in brains of mice following cerebral ischemia. **Blood 111:**5544–5552, 2008
50. Tsai MS, Hwang SM, Tsai YL, Cheng FC, Lee JL, Chang YJ: Clonal amniotic fluid-derived stem cells express characteristics of both mesenchymal and neural stem cells. **Biol Reprod 74:**545–551, 2006
51. Varejão AS, Cabrita AM, Meek MF, Bulas-Cruz J, Filipe VM, Gabriel RC, et al: Ankle kinematics to evaluate functional recovery in crushed rat sciatic nerve. **Muscle Nerve 27:**706–714, 2003
52. Varejão AS, Cabrita AM, Meek MF, Bulas-Cruz J, Melo-Pinto P, Raimondo S, et al: Functional and morphological assessment of a standardized rat sciatic nerve crush injury with a non-serrated clamp. **J Neurotrauma 21:**1652–1670, 2004
53. Wagers AJ, Sherwood RI, Christensen JL, Weissman IL: Little evidence for developmental plasticity of adult hematopoietic stem cells. **Science 297:**2256–2259, 2002
54. Weimann JM, Charlton CA, Brazelton TR, Hackman RC, Blau HM: Contribution of transplanted bone marrow cells to Purkinje neurons in human adult brains. **Proc Natl Acad Sci U S A 100:**2088–2093, 2003

Manuscript submitted September 10, 2010.
Accepted March 21, 2011.
Please include this information when citing this paper: published online August 19, 2011; DOI: 10.3171/2011.3.JNS101582.

Address correspondence to: Hung-Chuan Pan, M.D., Ph.D., Department of Neurosurgery, Taichung Veterans General Hospital, No. 160, Taichung-Kang Road, Section 3, Taichung 407, Taiwan. email: hcpan2003@yahoo.com.tw.