SUPPLEMENTARY INFORMATION

Functional Regeneration of Peripheral Nerve with Human Muscle-Derived Stem/Progenitor Cells

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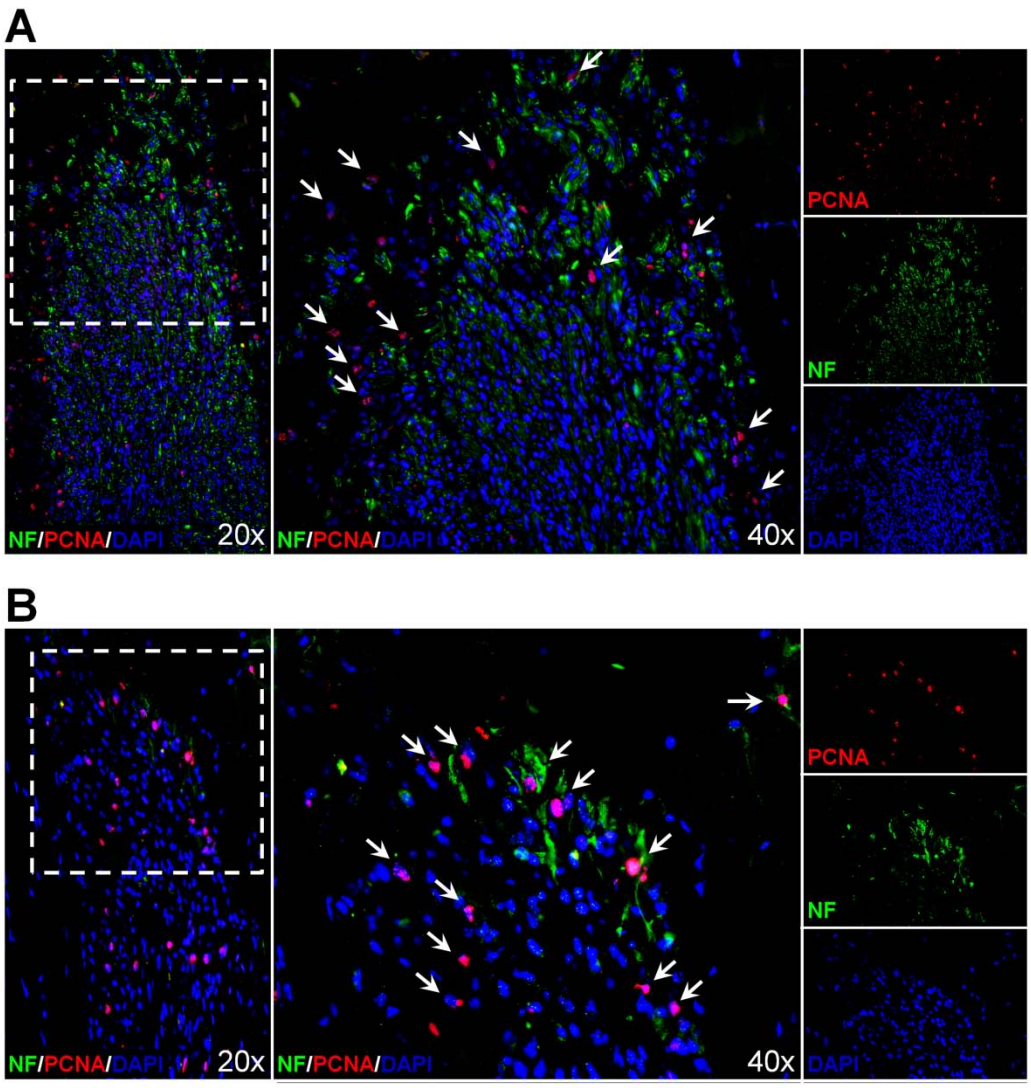
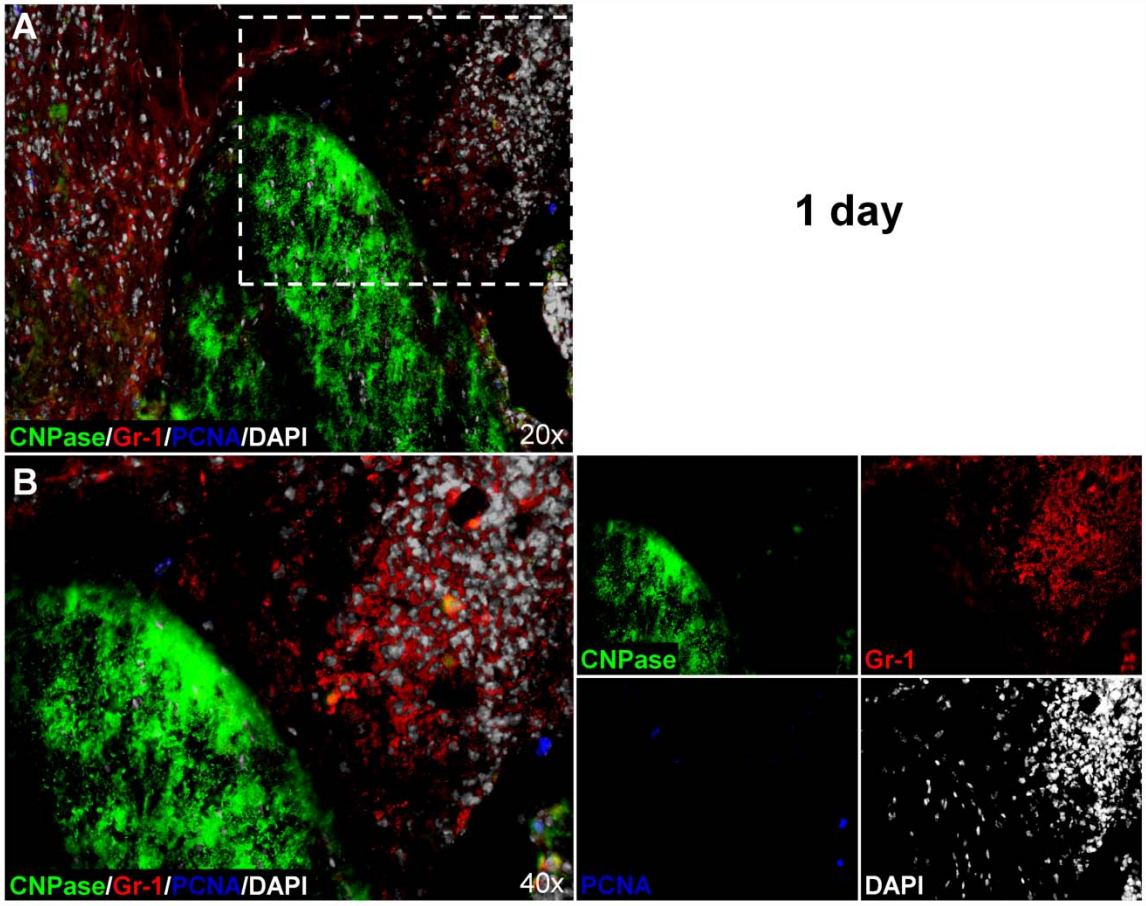
SUPPLEMENTARY FIGURES

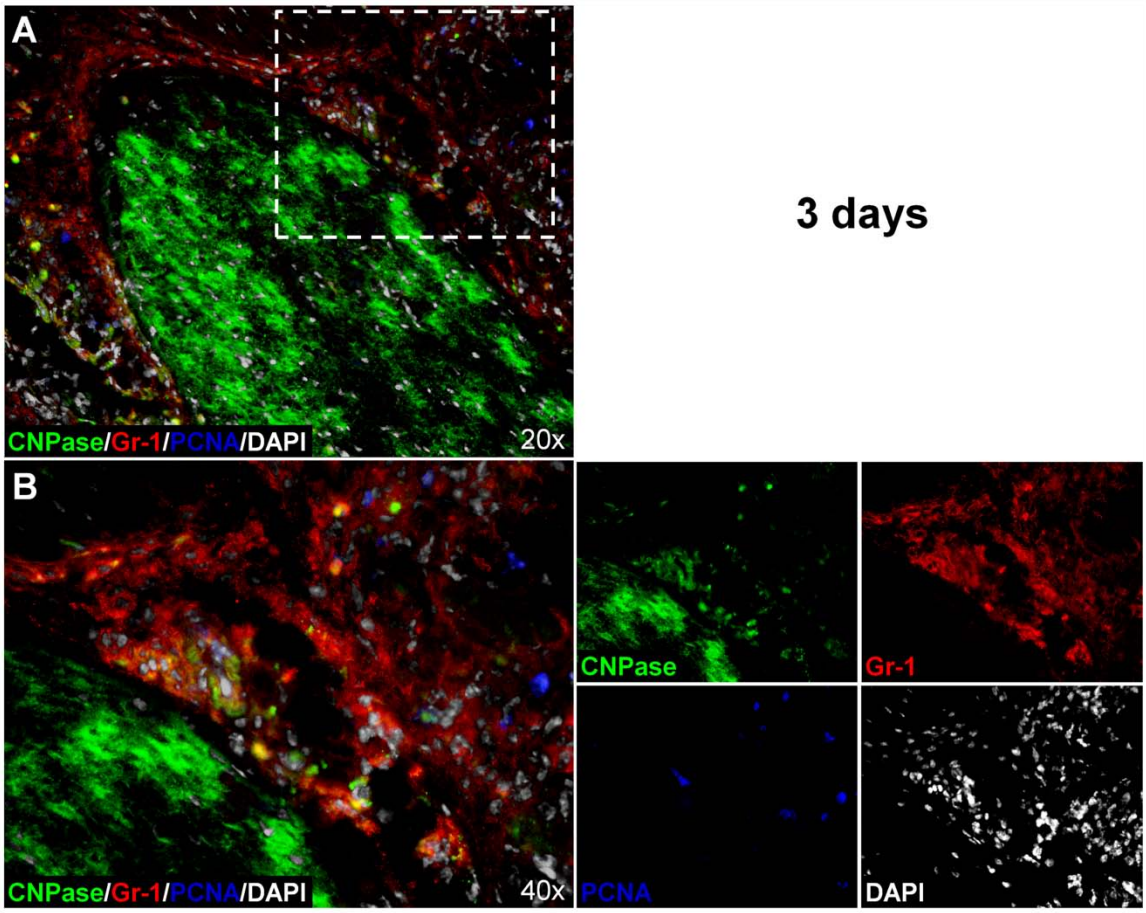
Supplementary Figure S1. To investigate the survival and engraftment of myoblasts after transplantation, $4x10^5$ rapidly adhering myoblast-like cells (pp3) were transplanted into a critical-sized sciatic nerve defect (~6.5 to 7 mm) of 6- to 8-wk-old SCID mice at the site of the proximal and distal nerve stumps immediately after injury (*n* = 18 mice, 3 independent myoblast populations). The hind limbs, including the sciatic nerves, were harvested, flash frozen in 2-methylbutane pre-cooled in liquid nitrogen, and serially sectioned at 6 µm thickness. Sections were immunostained for human-specific proliferating cell nuclear antigen (PCNA) to detect donor cells, neurofilament (NF) to reveal the axons, and stained with DAPI to identify the nuclei. (**A**) Representative images at 16 d post-transplantation showed human PCNA positive donor myoblasts surrounding and integrating (arrows) the growth-cone of the regenerating nerve, positive for NF. (**B**) Representative images at 4 wk indicate the presence of PCNA+ donor myoblasts; however, axonal degeneration was evident by loss of many NF+ axons.



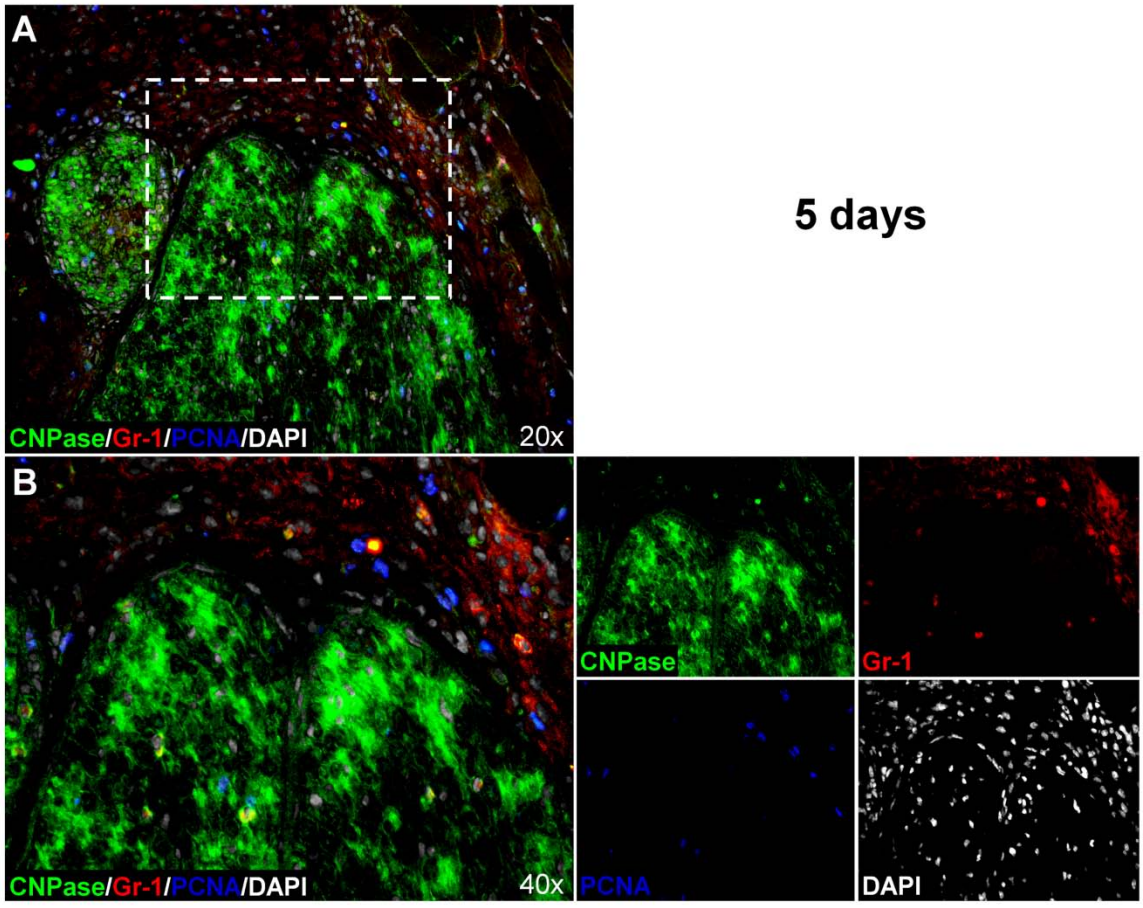
Supplementary Figure S2: To evaluate the degree of inflammation around the engraftment site post-injury and hMDSPC transplantation, mice (30 mice total, *n* = 3-6 mice per time point, 2 independent hMDSPC populations) were sacrificed at varying time points (1 d, 3 d, 5 d, 7 d, 16 d, 21 d, and 4 wk). Longitudinal cryosections of hindlimb, including the sciatic nerve, were immunostained for CNPase (green), Gr-1 (a marker for granulocytes and myeloid cells, red), and human specific PCNA (blue), as well as stained with DAPI (white) to identify nuclei of all cells. (**A**) Representative image of a triple immunostain of the growth cone including the surrounding tissues 1 d post-transplantation. (**B**) Shown are images of the dashed box in A at higher magnification, illustrating the extent of infiltration. Numerous Gr-1+ host neutrophils are shown infiltrating the area along and surrounding the CNPase+ growth cone of the regenerating nerve at 1 d following the hMDSPC transplantation.



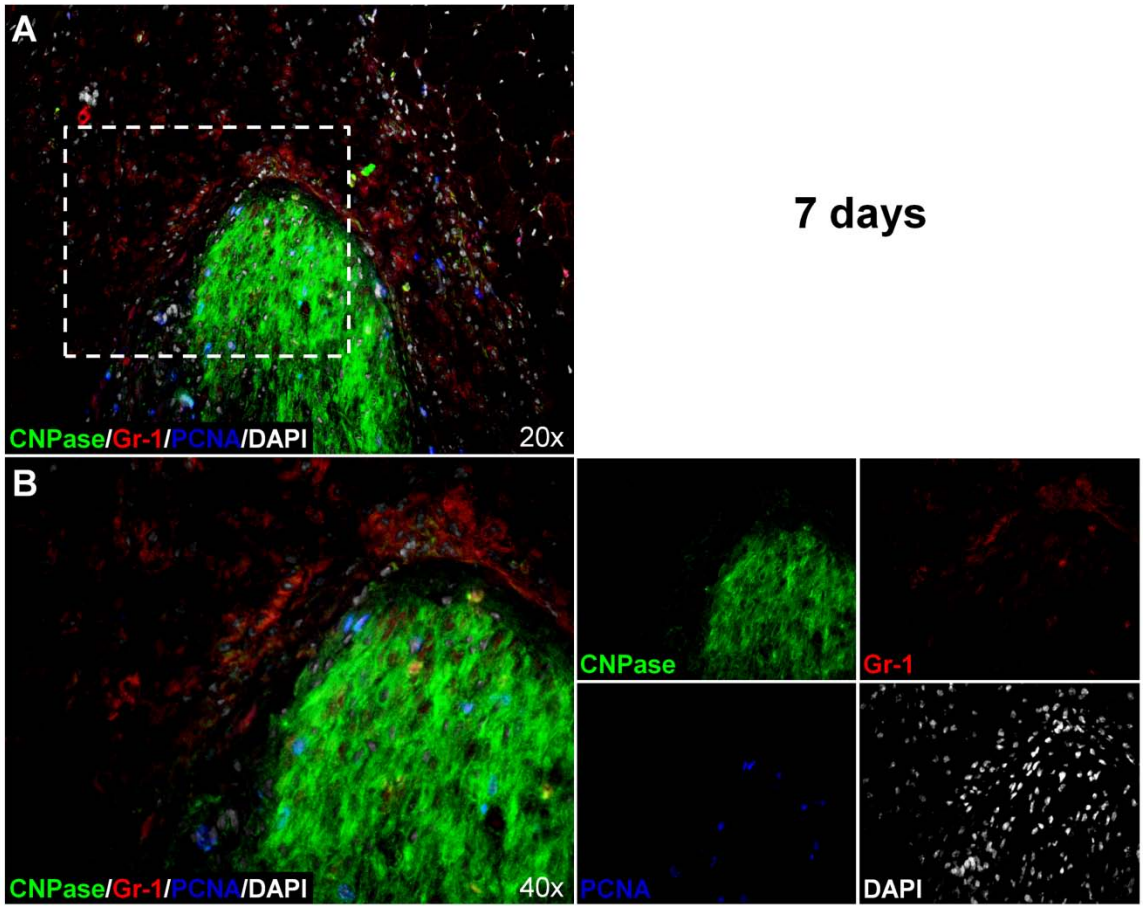
Supplementary Figure S3: Longitudinal cryosections of hindlimb, including the sciatic nerve, were immunostained for CNPase (green), Gr-1 (a marker for granulocytes and myeloid cells, red), and human specific PCNA (blue), as well as stained with DAPI (white) to identify nuclei of all cells. (**A**) Representative image of a triple immunostain of the growth cone including the surrounding tissues 3 d post-transplantation. (**B**) Shown are images of the dashed box in A at higher magnification illustrating the extent of infiltration. Many Gr-1+ host neutrophils are observed at the site of injury and along and surrounding the CNPase+ growth cone of the regenerating nerve 3 d following the hMDSPC transplantation.



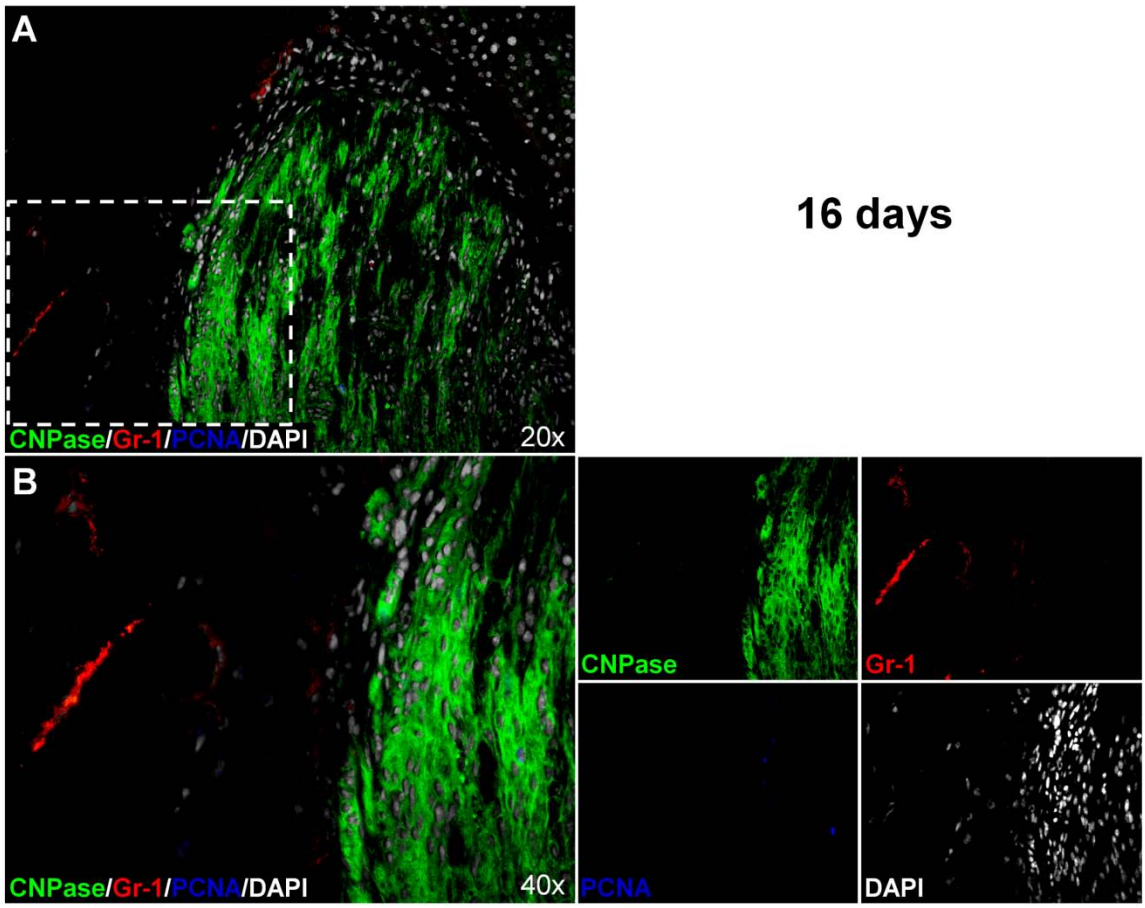
Supplementary Figure S4: Longitudinal cryosections of hindlimb, including the sciatic nerve, were immunostained for CNPase (green), Gr-1 (a marker for granulocytes and myeloid cells, red), and human specific PCNA (blue), as well as stained with DAPI (white) to identify nuclei of all cells. (**A**) Representative image of a triple immunostain of the growth cone including the surrounding tissues 5 d post-transplantation. (**B**) Shown are images of the dashed box in A at higher magnification illustrating the site and extent of infiltration. At 5 d following hMDSPC transplantation, a reduction in Gr-1+ neutrophils compared to previous time points is detectable along and surrounding the CNPase+ growth cone of the regenerating nerve.



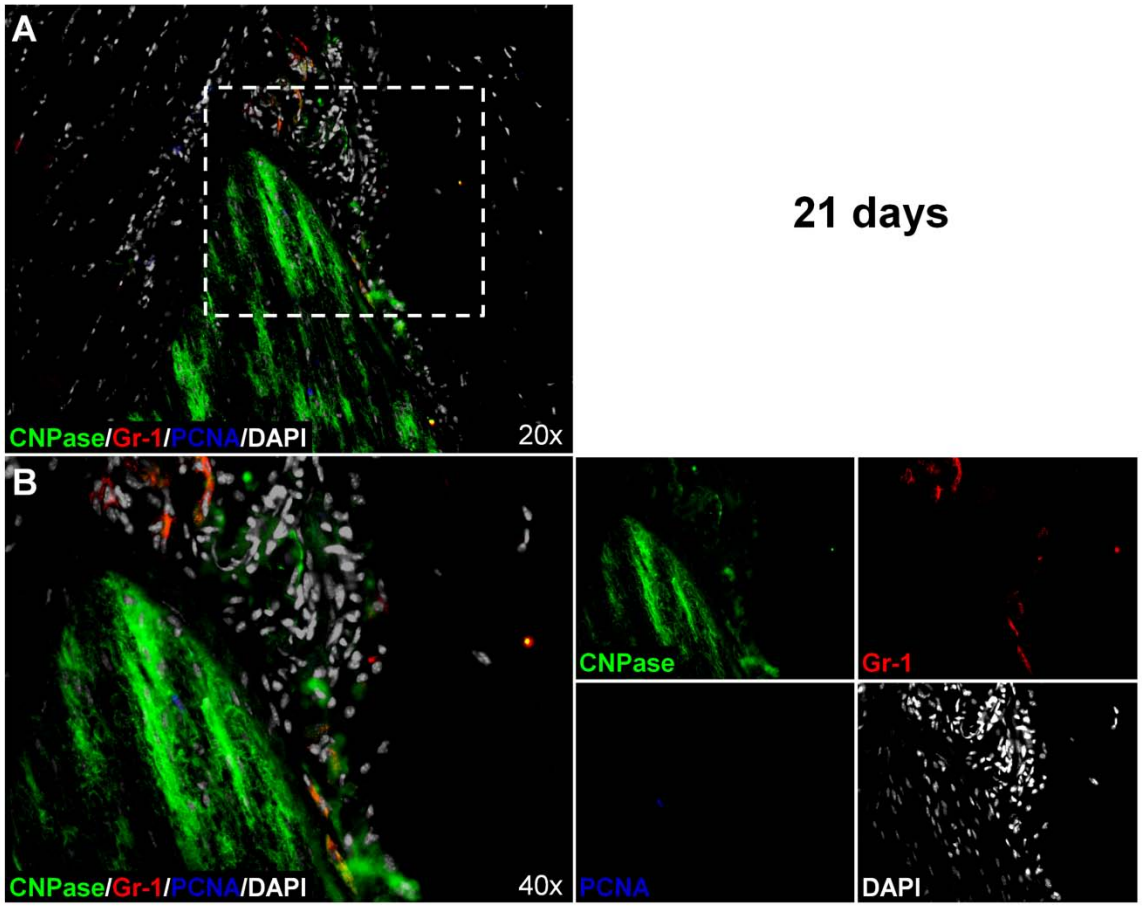
Supplementary Figure S5: Longitudinal cryosections of hindlimb, including the sciatic nerve, were immunostained for CNPase (green), Gr-1 (a marker for granulocytes and myeloid cells, red), and human specific PCNA (blue), as well as stained with DAPI (white) to identify nuclei of all cells. (**A**) Representative image of a triple immunostain of the growth cone including the surrounding tissues 7 d post-transplantation. (**B**) Shown are images of the dashed box in A at higher magnification illustrating the site and extent of infiltration. Host Gr-1+ neutrophils were still detectable at reduced levels along and surrounding the CNPase+ growth cone of the regenerating nerve 7 d following the hMDSPC transplantation.



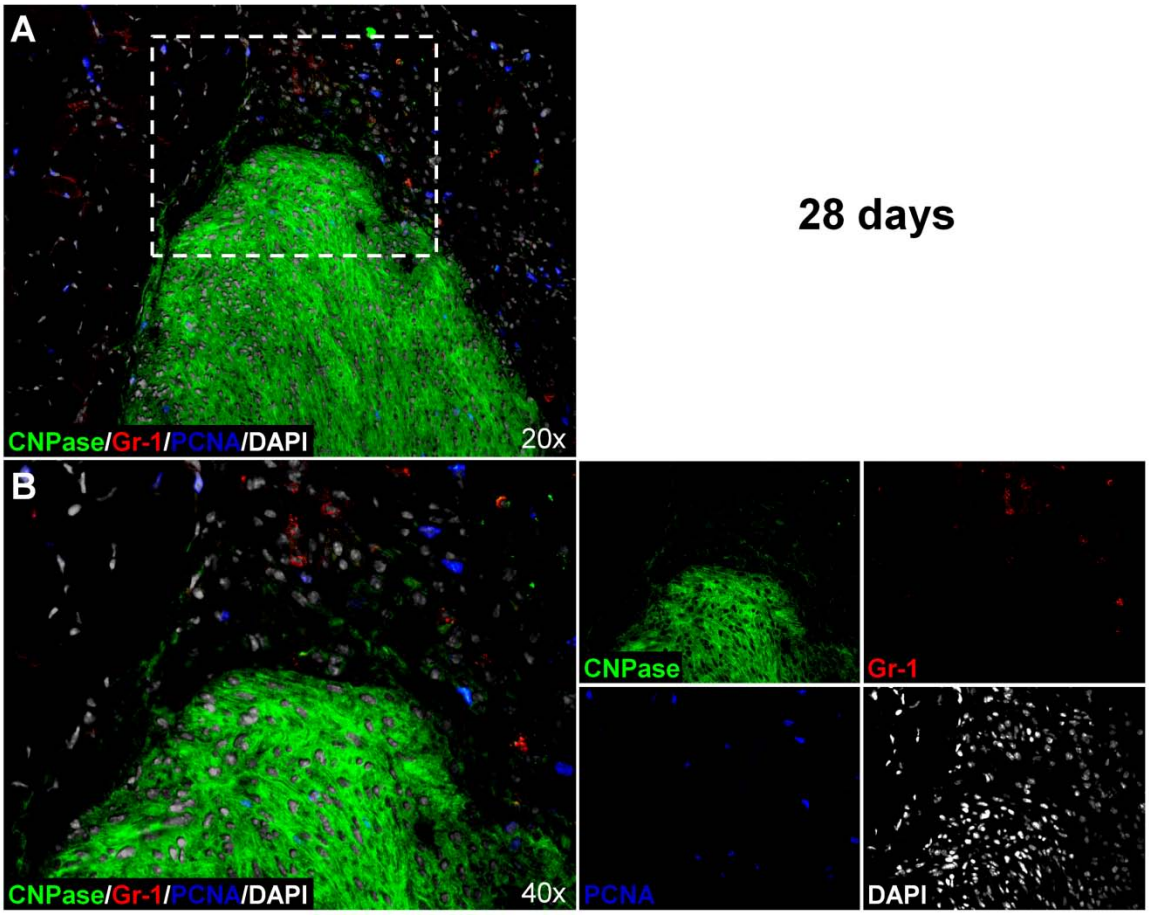
Supplementary Figure S6: Longitudinal cryosections of hindlimb, including the sciatic nerve, were immunostained for CNPase (green), Gr-1 (a marker for granulocytes and myeloid cells, red), and human specific PCNA (blue), as well as stained with DAPI (white) to identify nuclei of all cells. (**A**) Representative image of a triple immunostain of the growth cone including the surrounding tissues 16 d post-transplantation. (**B**) Shown are images of the dashed box in A at higher magnification illustrating the site and extent of infiltration. The number of Gr-1 positive host cells along and surrounding the CNPase+ growth cone of the regenerating nerve decreased noticeably 16 d following the hMDSPC transplantation.



Supplementary Figure S7: Longitudinal cryosections of hindlimb, including the sciatic nerve, were immunostained for CNPase (green), Gr-1 (a marker for granulocytes and myeloid cells, red), and human specific PCNA (blue), as well as stained with DAPI (white) to identify nuclei of all cells. (**A**) Representative image of a triple immunostain of the growth cone including the surrounding tissues 21 d post-transplantation. (**B**) Shown are images of the dashed box in A at higher magnification illustrating the site and extent of infiltration. Gr-1 positive neutrophils were hardly detectable at the growth cone of the regenerating nerve 21 d following the hMDSPC transplantation.



Supplementary Figure S8: Longitudinal cryosections of hindlimb, including the sciatic nerve, were immunostained for CNPase (green), Gr-1 (a marker for granulocytes and myeloid cells, red), and human specific PCNA (blue), as well as stained with DAPI (white) to identify nuclei of all cells. (**A**) Representative image of a triple immunostain of the growth cone including the surrounding tissues 28 d post-transplantation. (**B**) Shown are images of the dashed box in A at higher magnification illustrating the site and extent of infiltration. Twenty-eight d following the hMDSPC transplantation, very few Gr-1+ neutrophils are detectable at the engraftment site along and surrounding the CNPase+ growth cone of the regenerating nerve.



SUPPLEMENTARY METHODS

Neurosphere formation and differentiation

The formation of neurospheres was induced by plating hMDSPCs at a density of 1 x 10^5 cells / cm^2 on non-coated (Sigma-Aldrich) 24-well plates in NeuroCult NS-A Proliferation Medium (Human; STEMCELL Technologies) supplemented with human recombinant EGF, (20ng/ml; Invitrogen), human recombinant bFGF, (10ng/ml; Invitrogen), and 0.2% Heparin. To initiate differentiation, single suspensions of 7-8 d cultured hMDSPC-derived neurospheres (1.25 x 10^4 cells / cm^2) were plated on poly-D-lysine (Sigma-Aldrich) coated 8-well chamber slides (BD Falcon) in the NeuroCult NS-A Differentiation Medium (Human, STEMCELL Technologies). After 21-28 d, neuronal and glial differentiation was evaluated by immunocytochemical staining.

Immunocytochemistry

Four independent hMDSPC populations were screened for neuronal markers (NF, Tuj1, and MAP2), a synaptic vesicle protein marker (SV2A), and several glial and Schwann cell markers (Sox10, GFAP, S-100, p75, peripherin, and O4). The hMDSPCs were fixed for 20 min in 4% PFA (Fisher Scientific) made in PBS (pH 7.4; Invitrogen). Cells were washed and incubated for 5 min with PBS plus 0.3% Triton X-100 to permeabilize the cell membranes. Cells were then pre-incubated with 10% normal donkey serum (DS; Jackson ImmunoResearch Laboratories), to block non-specific binding, for 1 h at room temperature. Cells were incubated at 4°C overnight with the following primary antibodies (in 2.5% DS): NF (150 kD, 1:200, rabbit; Millipore), Tuj1 (1:500, rabbit; Sigma-Aldrich; or 1:500, mouse; Millipore), MAP2ab (1:500, mouse; Sigma-Aldrich), SV2A (1:500, rabbit; Synaptic Systems), Sox10 (1:500, rabbit; abcam), GFAP (1:500, rabbit; Millipore), S-100 (1:500, rabbit; Sigma-Aldrich), nerve growth factor receptor p75 (c-20, 1:50, goat; Santa Cruz Biotechnology), peripherin (1:300, rabbit; Millipore or 1:200, mouse; Millipore), and/or O4 (1:50, mouse; STEMCELL Technologies). The next day, the cells were

rinsed and exposed to the secondary antibodies of the appropriate species conjugated to Alexa Fluor® 594- or 488-conjugated (1:500; Molecular Probes), for 30 min. For negative controls, the primary Ab was omitted or replaced with mouse or rabbit IgG. To visualize the nuclei, the cultures were incubated with DAPI (100 ng/ml; Sigma-Aldrich) for 10 min. When using lamin A/C to label hMDSPCs, cells were incubated at 4°C overnight with lamin A/C Ab (1:200, mouse; Vector) combined with antibodies of interest, except others developed in mouse to avoid cross reactivity. The following day, cells were rinsed and incubated with biotinylated goat anti-mouse IgG (1:200, mouse; Vector) for 1 h at room temperature, and subsequently stained with streptavidin-conjugated with Alexa Fluor® 488 (for lamin A/C immunostaining, 1:500; Sigma-Aldrich) for 20 min. Culture slides were mounted with Vectashield® medium (Vector). All bright-field and fluorescent images were captured and processed using a Nikon Eclipse E800 microscope equipped with a Q imaging Retiga Exi digital camera or Leica DMIRB microscope equipped with a Q imaging Retiga digital camera, both using Northern Eclipse software (v. 6.0; Empix Imaging, Inc.). Cells immunostained for peripherin, p75, and/or Tuj1 were systematically quantified in at least 15 fields across the slides or wells of the culture dishes from 3 replica plating of 4 independent experiments. The ratio of positive nuclei to the total nuclei, detected by DAPI stain, defined the percent positive cells.

Real-time RT-PCR

The RNA from $1x10^5$ hMDSPCs (pre- or post-differentiation) isolated from 4 independent populations was extracted using a Nucleospin RNA kit (Clontech Laboratories). cDNA was synthesized with SuperScript™ II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The cDNA and primers, listed in Table 1, were added to SYBR green PCR master mix (Applied Biosystems) according to the manufacturer's instructions. All the primers were designed specifically against human genes and to span two different exons to avoid genomic DNA contamination. All target gene expressions were normalized to the

housekeeping gene Cyclophin as an internal control. The data were averaged and expressed as fold change relative to the values obtained from undifferentiated cells. All assays were carried out in triplicates.

Immunohistochemistry

Cryosections of the hind limb were first fixed in 4% PFA for 10 min, then counterstained with FluoroMyelin green (Molecular Probes), according to the protocol provided by the manufacturer, then blocked with 5% DS for 1 h, and incubated with primary Ab, anti-NF (1:200, rabbit; Millipore) for 2 h at room temperature. Tissues were then rinsed with PBS and stained with secondary Ab, donkey anti-rabbit Alexa Fluor® 594, for 20 min. In addition, the sciatic nerves were cryo-sectioned longitudinally, fixed with 5% formalin, blocked with 10% goat serum (GS), and incubated overnight at 4°C with both anti-NF (1:200, rabbit; Millipore) and anti-CNPase (1:200, mouse; Sigma-Aldrich) antibodies. The next day, the sections were rinsed with PBS, incubated with biotinylated goat anti-mouse IgG (1:200; Vector) for 1 h, and subsequently stained with streptavidin-conjugated Cy3 (1:200; Sigma-Aldrich) for CNPase immunostaining at room temperature for 20 min. The sections were then exposed to donkey anti-rabbit Alexa Fluor® 488 (1:500; Molecular Probes) for 20 min to immunostain for NF+ axons.

For human cell survival and colocalization studies, the sections were fixed, blocked with 5% GS, and incubated with an Ab against human-specific PCNA (1:500, human; US Biological), for 45 min. Sections were rinsed with PBS and exposed to goat anti-human Alexa Fluor® 555 Ab (1:500; Molecular Probes) for 15 min. Sections were then incubated with GFAP (1:500, rabbit; Chemicon) primary Ab for 3 h at room temperature, rinsed with PBS, and exposed to donkey anti-rabbit Alexa Fluor® 488 Ab (1:500; Molecular Probes) for 20 min.

For triple immunostaining, sections were fixed with 4% PFA for 10 min and stained using a Mouse on Mouse (M.O.M.) immunodetection kit (Vector Laboratories). Briefly, tissues were blocked with M.O.M.™ Mouse Ig Blocking Reagent for 1 h at room temperature. Following a 5

min incubation with M.O.M.™ Diluent, sections were incubated overnight at 4°C with anti-CNPase (1:200, mouse; Sigma-Aldrich), anti-human PCNA (1:500, human; US Biological), and anti-NF (1:200, rabbit; Millipore) Ab. The next day, the sections were rinsed with PBS and incubated with biotinylated horse anti-mouse IgG (1:250; Vector) for 15 min. This was followed by a 20 min incubation with streptavidin-conjugated 488 (1:200; Sigma-Aldrich) for CNPase, then a 30 min incubation with goat anti-human Alexa Fluor® 594 Ab (1:500; Molecular Probes) for PCNA, and finally a 30 min incubation with donkey anti-rabbit Alexa Fluor® 647 Ab (1:500; Molecular Probes) for NF detection, all at room temperature. All sections were stained with DAPI (100 ng/mL; Sigma-Aldrich) for 10 min at room temperature to identify cell nuclei. For negative controls, the primary Ab was omitted or replaced with mouse, rabbit, or rat IgG.

Inflammatory response

Randomly selected mice were sacrificed at varying time points [1 d (n = 3), 3 d (n = 3), 5 d (n = 3), 7 d (n = 3), 16 d (n = 6; 2 independent hMDSPC populations), 21 d (n = 6; 2 independent hMDSPC populations), 4 wk (n = 6; 2 independent hMDSPC populations)]. Longitudinal cryosections of hindlimbs, including the sciatic nerves, were immunostained for Ly6G and Ly6C (Gr-1), a marker for myeloid cells and granulocytes, following the protocol described above using the M.O.M. immunodetection kit (Vector Laboratories). Sections were incubated overnight at 4°C with anti-CNPase (1:200, mouse; Sigma-Aldrich), anti-Gr-1 (1:30, rat; BD Biosciences), and anti-human PCNA (1:500, human; US Biological) antibodies. Biotinylated horse anti-mouse, followed by streptavidin-conjugated 647, goat anti-rat Alexa Fluor® 488, and goat anti-human Alexa Fluor® 594, were used to detect CNPase, Gr-1, and PCNA, respectively. DAPI was used to identify cell nuclei.

Morphometric analysis of the regenerated nerve

The tissues were fixed in 2.5% glutaraldehyde (EM grade, Taab Chemical) in 0.1 M phosphate buffer (pH 7.3; Fisher Scientific) overnight at 4°C. The contralateral sciatic nerves harvested at the same level served as a “normal” control. The nerves were then rinsed in PBS, post-fixed in 1% osmium tetroxide (Electron Microscopy Sciences) with 0.1% potassium ferricyanide (Fisher Scientific), dehydrated in a series of ethanol solutions (30% - 90%; Fisher, and 100%; Pharmco), exposed to propylene oxide twice for 10 min each, and embedded in Polybed 812 (Dodecenyl Succinic Anhydride, Nadic Methyl Anhydride, Scipoxy 812 Resin and Dimethylaminomethyl; Energy Beam Sciences). All grafted segments were carefully oriented in order to obtain sections perpendicular to their long axis. Semi-thin (300 nm) sections were cut using a Leica Ultracut and stained with 0.5% toluidine blue (Fisher Scientific), examined under a light microscope (Olympus BX51) with Magnafire 2.1A image capture software, and quantified for the number of myelinated axons. The ultrathin sections (65 nm) were obtained at distal, mid, and proximal ends using a Leica Ultracut Microtome (Leica), stained with 2% uranyl acetate (Electron Microscopy Sciences) and lead citrate (Fisher Scientific), and examined through a JEOL JEM-1011 transmission electron microscope. The total number of myelinated axons was counted from ~10,000 μm^2 of the cross-sectional area of the nerve and median area of myelin thickness, median cross-sectional area of myelinated axons, and g-ratio (axonal area/myelinated fiber area) were examined in mid-sections. Each section was divided into four quarters. Each quarter of the nerve was analyzed individually, and morphometric parameters of >1500 fibers from hMDSPC group, (n = 3 mice, 3 independent populations) and >600 fibers in uninjured group, (n = 3 mice), respectively were calculated following image capture, background subtraction, image enhancement, automatic thresholding, and final editing.

Muscle reinnervation

The gastrocnemius muscles of injured (transplanted with hMDSPCs or PBS) and contralateral non-operated control legs were isolated and the wet weights were recorded. Collected data were normalized to the values of the non-operated control legs for each mouse and reported. The gastrocnemius muscles were then flash frozen in liquid-nitrogen-cooled 2-methylbutane, and serially sectioned (10 µm). Sections were fixed with 5% formalin and blocked with 10% DS in PBS for 1 h at room temperature, then incubated with rabbit anti-dystrophin Ab (1:1000, kindly provided by T. Partridge, Research Center for Genetic Medicine, Children's National Medical Center, Washington, DC) for 3 h. Sections were incubated with biotinylated anti-rabbit IgG for 1 h. Next, the sections were incubated with streptavidin-Cy3 (1:500, Sigma-Aldrich) for 20 min. Nuclei were revealed by the nuclear stain DAPI. Fluorescent images were captured with a Nikon Eclipse microscope and Northern Eclipse software was used to perform dimensional analysis of dystrophin+ myofibers. The images were thresholded using Northern Eclipse to distinguish the immunofluorescent signal from the back-ground noise signal, and then to determine the number and area of myofibers to provide quantitative measurements of the number of pixels occupied by each individual fiber. The fiber area distribution of >3,500 individual myofibers per group was measured by determining the total number of pixels occupied by each fiber and converted to square micrometers with Northern Eclipse.

For quantitation of postsynaptic AChR receptor densities, muscles were first stained for dystrophin following the same protocol as described above and then incubated with 0.2 µg/ml α-BTX Alexa Fluor® 488 (Invitrogen) in PBS for 45 min. The number of AChR clusters was counted manually and the dystrophin-myofibers were counted automatically as previously described using Northern Eclipse. The percent ratio of the AChR clusters to the total number of dystrophin+ myofibers in each image was calculated and averaged. To visualize the endplate AChR clusters, whole gastrocnemius muscles from 3-4 individual mice in each group were fixed with 2% PFA in PBS, rinsed with PBS, and labeled with α-BTX Alexa Fluor® 488 as described

above, Alexa Fluor® 647 Phalloidin (1:250, Invitrogen), and 0.001% Hoechst dye (bisBenzimide, Sigma) for 20 min. Three color, confocal stacks of motor end plates in the muscle were taken using an inverted Fluoview 1000 microscope (Olympus) and reconstructions were rendered using MetaMorph software (Molecular Devices).