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Glial Cell Line-Derived Neurotrophic Factor–Secreting Genetically Modified Human Bone Marrow-Derived Mesenchymal Stem Cells Promote Recovery in a Rat Model of Parkinson's Disease

Aleksandra Glavaski-Joksimovic,[1] Tamas Virag,[1] Thomas A. Mangatu,[1] Michael McGrogan,[2] Xue Song Wang,[1] and Martha C. Bohn[1]*

[1]Department of Pediatrics, Neurobiology Program, Children's Memorial Research Center, Feinberg School of Medicine, Northwestern University, Chicago, Illinois
[2]SanBio, Inc., Mountain View, California

Parkinson's disease (PD) is a neurodegenerative disease characterized by progressive degeneration of nigrostriatal dopaminergic (DA) neurons. The therapeutic potential of glial cell line-derived neurotrophic factor (GDNF), the most potent neurotrophic factor for DA neurons, has been demonstrated in many experimental models of PD. However, chronic delivery of GDNF to DA neurons in the brain remains an unmet challenge. Here, we report the effects of GDNF-releasing Notch-induced human bone marrow-derived mesenchymal stem cells (MSC) grafted into striatum of the 6-hydroxydopamine (6-OHDA) progressively lesioned rat model of PD. Human MSC, obtained from bone marrow aspirates of young, healthy adult volunteers, were transiently transfected with the intracellular domain of the Notch1 gene (NICD) to generate SB623 cells. SB623 cells expressing GDNF and/or humanized *Renilla* green fluorescent protein (hrGFP) following lentiviral transduction or nontransduced cells were stereotaxically placed into rat striatum 1 week after a unilateral partial 6-OHDA striatal lesion. At 4 weeks, rats that had received GDNF-transduced SB623 cells had significantly decreased amphetamine-induced rotation compared with control rats, although this effect was not observed in rats that received GFP-transduced or nontransduced SB623 cells. At 5 weeks, rejuvenated tyrosine hydroxylase-immunoreactive (TH-IR) fibers that appeared to be host DA axons were observed in and around grafts. This effect was more prominent in rats that received GDNF-secreting cells and was not observed in controls. These observations suggest that human bone-marrow derived MSC, genetically modified to secrete GDNF, hold potential as an allogeneic or autologous stem cell therapy for PD. **© 2010 Wiley-Liss, Inc.**

Key words: neurotrophic factor; gene therapy; nigrostriatal system; dopamine; Notch1

Parkinson disease (PD) is a neurodegenerative disease characterized by motor and postural deficits that occur as a consequence of a selective loss of dopaminergic (DA) neurons in midbrain and their projections to striatum. Current pharmacological and surgical therapies for PD ameliorate clinical symptoms, but do not halt or reverse the progressive DA neuronal death. Novel gene and stem cell therapies are being explored for PD to inhibit further degeneration of DA neurons or to replace lost DA neurons, respectively. Stem cells procured from various sources, including embryonic stem cells (ESC), neuroprogenitor cells isolated from fetal or adult brain, mesenchymal stem cells (MSC), human amniotic cells, and reprogrammed fibroblasts, have been shown to differentiate into DA neurons in vitro and in vivo following grafting (Sakurada et al., 1999; Kim et al., 2002;

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*Correspondence to: Martha C. Bohn, PhD, Medical Research Institute Council Professor, Director, Neurobiology Program, Children's Memorial Research Center (CMRC), Feinberg School of Medicine, Northwestern University, 2300 Children's Plaza, Box 209, Chicago, IL 60614. E-mail: m-bohn@northwestern.edu

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Dezawa et al., 2004; Yang et al., 2004; Roy et al., 2006; Kong et al., 2008; Muraoka et al., 2008; Barzilay et al., 2008; Wernig et al., 2008). In addition, several studies have revealed beneficial effects of grafting stem cells and neuroprogenitors in experimental models of PD (Kim et al., 2002; Dezawa et al., 2004; Behrstock et al., 2006; Shintani et al., 2007; Wang et al., 2007; Bouchez et al., 2008; Kong et al., 2008; Geeta et al., 2008; Wernig et al., 2008). In the present study, we explore an alternative strategy through which stem cells are exploited to promote recovery of damaged host DA neurons via secretion of neurotrophic factors.

Glial cell line-derived neurotrophic factor (GDNF), a factor isolated from the B49 cell line, was found to have an extremely potent effect on survival, growth, and function of DA neurons cultured from the embryonic rat brain (Engele et al., 1991; Lin et al., 1993). Subsequently, neuroprotective effects of GDNF protein were demonstrated in an array of rodent and primate models of PD (Hoffer et al., 1994; Beck et al., 1995; Sauer et al., 1995; Tomac et al., 1995; Gash et al., 1996). Several clinical trials using GDNF protein were undertaken based on these promising effects, but these were subsequently stopped because of the difficulty of targeting GDNF protein specifically to the DA neurons without eliciting side effects (Nutt et al., 2003; Lang et al., 2006). The field has now progressed to examining gene therapy approaches to overcome the brain–blood barrier and protein delivery problems. Several groups have reported that chronic delivery of very low levels of GDNF via injection of various types of gene therapy viral vectors, including adenovirus, adeno-associated virus, herpes simplex virus, and lentivirus, protect and/or rescue DA neurons in rodent and primate models of PD (Choi-Lundberg et al., 1997, 1998; Connor et al., 1999; Bjorklund et al., 2000; Kordower et al., 2000; Kozlowski et al., 2000; Natsume et al., 2001; Eberling et al., 2009; Johnston et al., 2009). In addition to the use of viruses for direct gene delivery are ex vivo GDNF gene delivery approaches using GDNF gene transfer to various cell types in culture, followed by transplantation into brain (Akerud et al., 2001; Park et al., 2001; Cunningham and Su, 2002; Ericson et al., 2005), and recently human neural stem cells (NSC; Behrstock et al., 2006, 2008; Emborg et al., 2008). In the present study, for the first time, Notch-induced human bone marrow-derived MSC, termed *SB623 cells* (SanBio, Inc., Mountain View, CA), were tested for this purpose. MSC derived from human bone marrow, also referred to as multipotent stromal cells, are excellent candidates for cell therapies insofar as they are readily available and lack the ethical issues associated with ESC or neuroprogenitor cells of human fetal origin. In addition, several in vitro and in vivo studies have demonstrated multilineage differentiation of MSC, including differentiation into cells with morphological and phenotypic characteristics of glia and neurons (Kopen et al., 1999; Pittenger et al., 1999; Woodbury et al., 2000; Sanchez-Ramos, 2002; Verfaillie, 2002; Jiang et al., 2002; Dezawa et al., 2004; Bertani et al., 2005; Dezawa, 2006; Choong et al., 2007; Alexanian et al., 2008; Barzilay et al., 2008; Bahat-Stroomza et al., 2009). There is also growing evidence that transplantation of MSC promotes recovery in different animal models of neuronal injury and neurodegenerative disorders, including PD, stroke, and spinal cord injury (Dezawa et al., 2001, 2004; Li et al., 2001; Chopp and Li, 2002; Hofstetter et al., 2002; Zhao et al., 2002; Ankeny et al., 2004; Borlongan et al., 2004; Cizkova et al., 2006; Offen et al., 2007; Shintani et al., 2007; Tang et al., 2007; Bouchez et al., 2008; Park et al., 2008; Hayase et al., 2009; Yasuhara et al., 2009). We recently reported that SB623 cells evoke rejuvenation of host DA fibers in striatum of 6-hydroxydopamine (6-OHDA) partially lesioned rats (Glavaski-Joksimovic et al., 2009). Here, we expand on this finding and examine the potential of these cells for delivering GDNF into striatum and evoking recovery of host DA neurons. Adherent bone marrow stromal cells isolated from human aspirates were transiently transfected with a plasmid encoding NICD to generate SB623 cells. SB623 cells were then transduced with a self-inactivating, helper-free lentiviral vector to express GDNF and/or humanized *Renilla* green fluorescent protein (hrGFP). Transduced or nontransduced SB623 cells were grafted at two sites in rat striatum 1 week after a striatal 6-OHDA lesion. The control rats were lesioned and injected with the same volume of PBS using the same coordinates or were assigned to a lesion-only group. The survival of grafted cells and their effects on 6-OHDA-damaged DA neurons were assessed by immunocytochemistry, morphometry, and amphetamine-induced rotation.

MATERIALS AND METHODS

Genetically Modified Human MSC-SB623 Cells

SB623 cells have been previously characterized (Dezawa et al., 2004; Mimura et al., 2005; Aizman et al., 2009) and were provided by SanBio, Inc. (Mountain View, CA). Human adult bone marrow aspirates (Lonza, Walkersville, MD) were washed once and plated in Corning T225 flasks (Corning, Inc., Lowell, MA) in α-minimum essential medium (αMEM; Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 2 mM L-glutamine, and penicillin/streptomycin (both from Gibco, Carlsbad, CA). After 3 days, unattached cells were removed; the adherent MSC cultures were maintained in growth medium changes for about 2 weeks and passed using 0.25% trypsin/EDTA (Gibco). Transient transfection of MSC cells with pCI Neo plasmid containing human NICD (amino acids 1703–2504) and neomycin-resistance gene was performed using Fugene6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's protocol. Briefly, cells were transfected with the Fugene6 plasmid DNA complex for 24 hr and then selected in G418 (100 mg/ml; Gibco) for 7 days. After removal of G418 selection media, cultures were expanded, harvested using trypsin/EDTA (Gibco), and cryopreserved.

The results from fluorescence-activated cell sorting (FACS) of the MSC, used for generation of SB623 cells, show that they express high levels of CD29, CD73, CD90, and CD105, but do not express detectable levels of the hematopoietic markers CD31, CD34, and CD45, confirming their

mesenchymal origin. These MSC cells are pluripotent and can be differentiated into chondrocytes, adipocytes, and osteocytes when properly induced. The resulting SB623 cells share many of the markers with the parental MSC cells, but show a reduced potential to differentiate and enhance expression of neurotrophic factors.

Lentiviral Vectors (Lenti)

HIV-based, replication-incompetent, self-inactivating lentiviruses were obtained from the Children's Memorial Research Center (CMRC) viral vector core. Lenti-CMV-GDNF-IRES-hrGFP harbors human GDNF and hrGFP under control of the cytomegalovirus (CMV) immediate early promoter and contains an internal ribosome entry site (IRES). Lenti-CMV-hrGFP was the control vector. These lentiviruses were packaged using a four-plasmid system and purified using standard procedures as described previously, with minor modifications (Zufferey, 2002; Sapru et al., 2006). The viral titers were 3×10^8 transducing units (TU)/ml for Lenti-CMV-hGDNF-IRES-hrGFP and 2×10^8 TU/ml for Lenti-CMV-hrGFP.

Lentivirus Infection of SB623 Cells

Thawed cells were washed once with α-MEM medium (Lonza) supplemented with 10% FBS (Gibco) and 1% penicillin/streptomycin (Gibco), and viable cells as determined by trypan blue exclusion were plated at a density of 7×10^5 cells/100-mm dish. One day later, cells were infected with Lenti-CMV-hGDNF-IRES-hrGFP or Lenti-CMV-hrGFP at a multiplicity of infection (MOI) of 5 or 10. Four days after infection, virus-containing medium was removed, and the cells were washed once with α-MEM (Lonza) supplemented with 1% penicillin/streptomycin (Gibco). Transduced SB623 cells were expanded for transplantation, and an additional set of untransduced cells was expanded for a control.

Measurements of GDNF Secretion In Vitro

The amount of GDNF secreted into the medium from SB623 cells was measured 72 hr after transduction using the human GDNF-ELISA DuoSet (R&D Systems Inc., Minneapolis, MN) according to the manufacturer's instructions. Data are expressed in picograms per milliliter.

SB623 Cell Preparation for Transplantation

At 7 days in culture, transduced and nontransduced SB623 cells were harvested for grafting by trypsinization for 5 min at 37°C (0.25% trypsin/EDTA; Gibco). Trypsin was inactivated by addition of 10 ml α-MEM medium supplemented with 10% FBS. Cells were then pelleted, resuspended in Dulbecco's phosphate-buffered saline without calcium and magnesium (DPBS w/o Ca^{2+}-Mg^{2+}; Hyclone), and counted. For grafting, cells were again pelleted, buffer was removed, and cell counting was done to confirm the final concentration and viability prior to implantation. Cell viability was also verified after grafting.

Animals

In total, 67 adult Fisher 344 (F344) male rats (200–300 g; Harlan, Indianapolis, IN) were used in two independent experiments. Rats were housed in the CMRC vivarium in a standard 12-hr light/dark cycle with food and water available ad libitum. All animal-related procedures were conducted in accordance with institutional, USDA, and NIH guidelines.

Lesion and Transplantation Surgery

6-OHDA lesion. Before 6-OHDA injection, baseline data for amphetamine-induced rotations were collected on all rats, as described below. Fifty-three F344 rats were divided among the five experimental groups (nontransduced SB623 cells, n = 12; hrGFP-transduced SB623 cells, n = 12; GDNF/hrGFP-transduced SB263 cells, n = 12; vehicle control, n = 12; lesion only, n = 5) for equal mean rotation per group, and the hemisphere for 6-OHDA lesion was assigned contralateral to the natural rotation bias. Rats, while under isofluorane anesthesia, received a partial, progressive lesion of striatal DA axons by injection of 6-OHDA unilaterally into the striatum +0.2 AP and ±3.0 ML from Bregma (Sauer and Oertel, 1994; Choi-Lundberg et al., 1997) using a digital Quintessential Stereotaxic Injector attached to a Stoelting stereotaxic apparatus (Stoelting Co., Wood Dale, IL). Breathing and corneal and toe-pinch responses were continuously monitored. A burr hole was made unilaterally. A 26-G injection needle attached to a 10-μl Hamilton syringe was lowered to –5.0 DV at a rate of 1 mm/min and held in place for 2 min before starting the injection. The lesion was made by injecting 2.8 μl saline containing 0.2 mg/ml ascorbic acid and 16 μg 6-OHDA-HBr (Sigma, St. Louis, MO) at a rate of 0.5 μl/min. After injection, the needle was held in place for 5 min and then retracted at 1 mm/min. The skin was sutured using discontinuous stitches, and double antimicrobial ointment (Fougera, Melville, NY) and LMX4 topical anesthetic (Ferndale Laboratories Inc., Ferndale, MI) were used on the surgical site.

SB623 cell grafting in normal striatum. In a pilot experiment to test cell survival, SB623 cells transduced with lenti-CMV-hGDNF-IRES-hrGFP were stereotaxically placed in the right, nonlesioned striatum of 14 adult F344 rats. Six rats received SB623 cells transduced with lentivirus at 10 MOI, and eight rats received SB623 cells transduced at 5 MOI. Each rat received two deposits of 2 μl of cell suspension adjusted to a concentration of 30,000 cells/μl in phosphate-buffered saline (PBS). Survival was for 1 (n = 4), 2 (n = 5), or 4 (n = 5) weeks postgrafting.

In a second experiment, rats were grafted with nontransduced, hrGFP-transduced, or GDNF/hrGFP-transduced SB623 cells 1 week after a 6-OHDA lesion. Two deposits of 2 μl SB623 cells suspension in PBS adjusted to concentration of 30,000 cells/μl were stereotaxically injected in the lesion striatum at +0.2 AP, ±3.0 ML, –4.4/5.8 DV. Control rats received PBS in the same volume and at the same coordinates or were assigned to a lesion-only group. The same general methods were used as in the 6-OHDA surgeries, but the injection protocol was changed to accommodate cell injections. Injection of cells (2 μl/deposit, 1 μl/min) was done as

described above using a 26-G needle (30° angle tip) with the needle held in place for 2 min before and after injection.

All rats were immunosuppressed starting 24 hr prior to cell grafting and daily thereafter by subcutaneous injections of cyclosporine (Sandimmune 10 mg/ml in saline; Novartis Pharmaceuticals, East Hanover, NJ). Rats were euthanized at 5 weeks postgrafting by transcardial perfusion with 0.9% saline followed by 4% paraformaldehyde in PBS while anesthetized with Nembutal (50 mg/kg).

Tissue Processing

Tissue blocks containing striatum were postfixed overnight and then crytoprotected in a series of 10%, 20%, and 30% sucrose. Frozen coronal sections (40 μm) were made and kept in a cryoprotective solution at –20°C until stained.

Histology

Tyrosine hydroxylase immunoreactivity. Sections were washed three times in PBS, incubated for 15 min in 0.3% H_2O_2 in PBS, blocked for 20 min at room temperature (RT) in 10% normal goat serum (NGS)-0.5% Triton X-100 (TX-100; Sigma) in PBS, and briefly washed in PBS/0.1% TX-100. Sections were then incubated overnight at RT on an orbital shaker with 1:2,000 polyclonal rabbit anti-TH antibody (Millipore-Chemicon, Billerica, MA) in 1% NGS and 0.3% TX-100 in PBS. Sections were then washed briefly in PBS/0.1% TX-100 and incubated for 2.5 hr at RT with biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA) diluted 1:500 in 1% NGS and 0.1% TX-100 in PBS, followed by incubation in avidin-peroxidase conjugate (Vectastain ABC Elite, Vector Laboratories) in PBS for 2 hr. Visualization was done with 20 mg/ml diaminobenzidine (DAB; Sigma), 0.8% nickel sulfate, 0.005% H_2O_2 in 50 mM sodium acetate, 10 mM imidazole buffer (pH 7.0).

Immunofluorescence. Sections and SB623 cells fixed with 4% paraformaldehyde were washed several times in PBS, blocked for 1 hr at RT in 5% normal serum (goat or donkey) and 5% bovine serum albumin (BSA) in PBS with 1% TX-100, and then incubated in primary antibody for 48 hr at 4°C. Primary antibodies were diluted in a blocking solution as follows: mouse anti-human nuclear antigen (hNA; Millipore-Chemicon) 1:50, mouse anti-human nuclear mitotic apparatus protein (hNuMA; Calbiochem, San Diego, CA) 1:50, goat anti-human Endoglin (CD105; R&D Systems) 1:50, mouse antinestin human specific (Millipore-Chemicon) 1:100, neuron-specific nuclear protein NeuN (Millipore-Chemicon) 1:100. For hNuMA staining, first antigen retrieval was performed by transferring the striatal sections into sodium citrate buffer (10 mM, pH 8.5, at 80°C for 30 min). After incubation with primary antibody, sections were washed several times with 0.02% TX-100 in PBS, and the signal was detected by incubation for 90 min at RT with an appropriate IgG conjugated to Cy3 (Jackson Immunoresearch, West Grove, PA) diluted 1:250 in a blocking solution without TX-100. Hoechst nuclear staining was done by treating sections with Hoechst 33,342 dye (5 μg/ml in PBS; Gibco) for 15 min. Staining specificity was confirmed by omission of the primary antibody.

For double labeling against hNuMa and TH, mouse monoclonal anti-hNuMA antibody (1:50, Calbiochem) was applied simultaneously with TH polyclonal rabbit antibody (1:500, Millipore-Chemicon). Visualization was performed by incubating sections for 90 min at RT with secondary antibodies conjugated to Cy3 or Cy2 (Jackson Immunoresearch) diluted 1:250 in a blocking solution without TX-100. Hoechst staining was then performed as described above.

Immunostained sections and SB623 cells were coverslipped with FluorSave mounting media (Santa Cruz Biotechnology, Santa Cruz, CA) and analyzed using a Leica DMR upright fluorescent microscope (Leica Mycrosystems, Wetzlar, Germany) equipped with a Retiga 4000R Fast 1394 digital CCD camera (QImaging, Burnaby, British Columbia, Canada).

TH-IR Fibers Density

The quantitation of TH-IR fiber optical density (OD) was performed with a slight modification of previously described methods (Jollivet et al., 2004; Garbayo et al., 2009). For each rat, the density of TH-IR fibers was measured at three different rostrocaudal levels. The fiber density in the medial striatum at the level of the grafts was taken as the mean of the density at the AP levels +0.2 and 0.0 mm relative to Bregma, and the OD of the caudal striatum from AP level –1.0 mm in the globus pallidus. For each section, images at corresponding levels in the control, nonlesioned side were also acquired. All images (2,048 × 2,048 pixels) were taken at ×100 with the same settings and saved as tiff files. Brown cells in the graft site, which were most likely dead cells or macrophages, were removed in Adobe Photoshop CS4 using the Color Replacement or Eraser tool. After removing the brown cells, new images were compared with the original ones to verify that black TH-IR fibers had not been removed and that most brown background/cells has been removed. Images were saved as uncompressed eight-bit gray-scale tiff files and exported to ImageJ software (NIH, Bethesda, MD). In ImageJ, the background was subtracted using the same setting for all images in a data set. The best setting for background subtraction was determined empirically by testing on images with the lowest and highest amounts of TH-IR fibers. Furthermore, the threshold intensity that provided an image closest to the original for TH-IR fibers was determined, and the same value was used to threshold and generate binary images for all images in a data set. Thereafter, particle analysis was carried out using the Analyze Particles tool (Grider et al., 2006). The area fraction covered with TH-IR fibers on the lesoned side was expressed as a percentage of that on the contralateral, nonlesioned side.

Behavioral Testing: Amphetamine-Induced Rotation

Rotational behavior induced by an i.p. injection of 5 mg/ml *d,l*-amphetamine sulfate was assessed using a computerized rotometer (Rotomax; AccuScan Instruments, Inc., Columbus, OH) prior to the 6-OHDA surgery to establish a baseline and assign a surgery side (Ungerstedt, 1971; Choi-Lundberg et al., 1998; Kozlowski et al., 2000). Treatment groups were then assigned to have equivalent baseline rotations. However, one additional rat was later added to the ex-

perimental group that received GFP-transduced SB623 cells, to compensate for rats that did not meet criteria. This slightly increased the baseline data for this group, but did not result in a statistically significant difference at time zero. Amphetamine-induced rotation was again assessed 1 day before and at 2 and 4 weeks after cell grafting. The numbers of clockwise and counterclockwise turns were recorded during an 80-min period, and the first 20 min was excluded. Rotational scores were expressed as net ipsilateral turns/min. Only rats exhibiting a net rotational asymmetry of at least six full ipsiversive turns/min at 6 days after lesion (1 day before grafting; n = 36) were included for further study.

Forelimb Use for Weight-Bearing Movement

Forelimb use during exploration of vertical surfaces and landing was analyzed as previously described (Connor et al., 1999; Kozlowski et al., 2000). Before the lesion, 6 days postlesion (just prior to grafting), and 4 weeks postgrafting, rats were placed in a plexiglass cylinder and videotaped for 5 min. The numbers of ipsilateral (to the lesion), contralateral, or both paw placements performed against the chamber walls were scored by a blinded observer during vertical/lateral explorations and during landing on the floor after termination of wall contact. The percentages of ipsilateral forelimb wall use (ipsilateral wall/ipsilateral + contralateral + both wall behaviors) and ipsilateral forelimb land use (land/ipsilateral + contralateral + both land behaviors) were calculated separately. These two scores were added and divided by 2 to obtain the percentage of total ipsilateral forelimb use. At each time point, rats were excluded from analyses if they exhibited fewer than five forelimb placements or if they were removed from the amphetamine-induced rotation analysis.

Statistical Analysis

All data were analyzed in GraphPad Prism software and expressed as mean ± SEM. Morphological and weight data were analyzed by using a one-way ANOVA for treatment with post hoc analysis conducted using Tukey's test. Behavioral data were analyzed with a repeated-measures two-way ANOVA for treatment and time of testing, with time being the repeated measure. Post hoc analyses were conducted using the nonparametric Mann Whitney test. Significant differences were taken at $P = 0.05$. For morphological and behavioral data analyses, PBS-injected and lesion-only rats were pooled, after determining that there was no significant difference between these groups.

RESULTS

Lentiviral Transduction of SB623 Cells With GDNF and/or hrGFP

To test whether SB623 cells can be used to deliver GDNF to the rat brain, secretion of GDNF from SB623 cells following lentiviral transduction was studied. Cells were infected with Lenti-CMV-GDNF-IRES-hrGFP or Lenti-CMV-hrGFP. Transduction efficiency was very high, and at 3 days more than 95% of SB623 cells that were labeled with hNA also displayed cytoplasmic GFP fluorescence (Fig. 1A–C). At the same time, conditioned medium was analyzed by ELISA to determine levels of GDNF secretion (Fig. 1D). SB623 cells transduced with lentivirus at three different MOIs (1, 5, or 10) secreted high levels of GDNF into the medium (Fig. 1D). The samples were diluted 100-fold to bring them down to the linear range of the standards (Fig. 1D).

Survival of GDNF-Secreting SB623 Cells in Nonlesioned Rat Striatum

In a pilot experiment, rats received grafts of Lenti-CMV-hGDNF-IRES-hrGFP-transduced SB623 cells in nonlesioned stratium. At 1, 2, and 4 weeks postgrafting, cell survival was analyzed. At 1 week postgrafting, numerous surviving cells were observed in all rats (Fig. 1E). At 2 weeks postgrafting, the number of surviving cells was significantly reduced (Fig. 1F,G), and, at 4 weeks, only GFP-fluorescent cell debris was observed in graft sites (Fig. 1H). At 2 weeks postgrafting, slightly better survival was observed in rats that had received SB623 cells transduced at 5 MOI (Fig. 1F) compared with those grafted with cells transduced at 10 MOI (Fig. 1G), so 5-MOI-transduced SB623 cells were used for subsequent studies.

At 1 week postgrafting, surviving cells in graft sites were visualized by their GFP fluorescence and by immunoreactivity (IR) to hNuMA (Fig. 1I–K). The majority of surviving cells was observed in a dorsoventral direction along the needle track, although a few grafted cells had migrated into the host parenchyma and were observed just outside the main core of the graft (Fig. 1I–K). The phenotype of surviving cells was also examined at 1 week postgrafting. The majority of surviving, GFP-fluorescent cells at the graft site expressed the MSC cell marker endoglin (Supp. Info. Fig. 1A–C). A smaller number of surviving cells expressed nestin (Supp. Info. Fig. 1D–F), an intermediate filament protein considered primarily as a neuroprogenitor marker (Lendahl et al., 1990), but which can also be expressed in endothelial cells (Mokry et al., 2008). However, grafted GFP-fluorescent cells were not colabeled with the mature neuronal marker NeuN, suggesting that NeuN-IR cells at the graft site were of host origin (Supp. Info. Fig. 1G–I).

Transgene GDNF protein expression in the nonlesioned rat striatum was examined using immunostaining against human GDNF. At 1 week postgrafting, strong GDNF-IR was observed at the graft site (Fig. 1L,M). The GDNF-IR penumbra surrounding the graft site likely represents the area over which GDNF was secreted from the grafted cells (Fig. 1L,M). However, at 2 weeks postgrafting, GDNF-IR was observed only within the graft site (Fig 1N,O), which correlates with the observed decrease in the number of surviving cells at this time point.

No Effect of GDNF-Secreting Cells on Body Weight

Grafting of transduced and nontransduced SB623 cells into the lesioned striatum was well tolerated by the

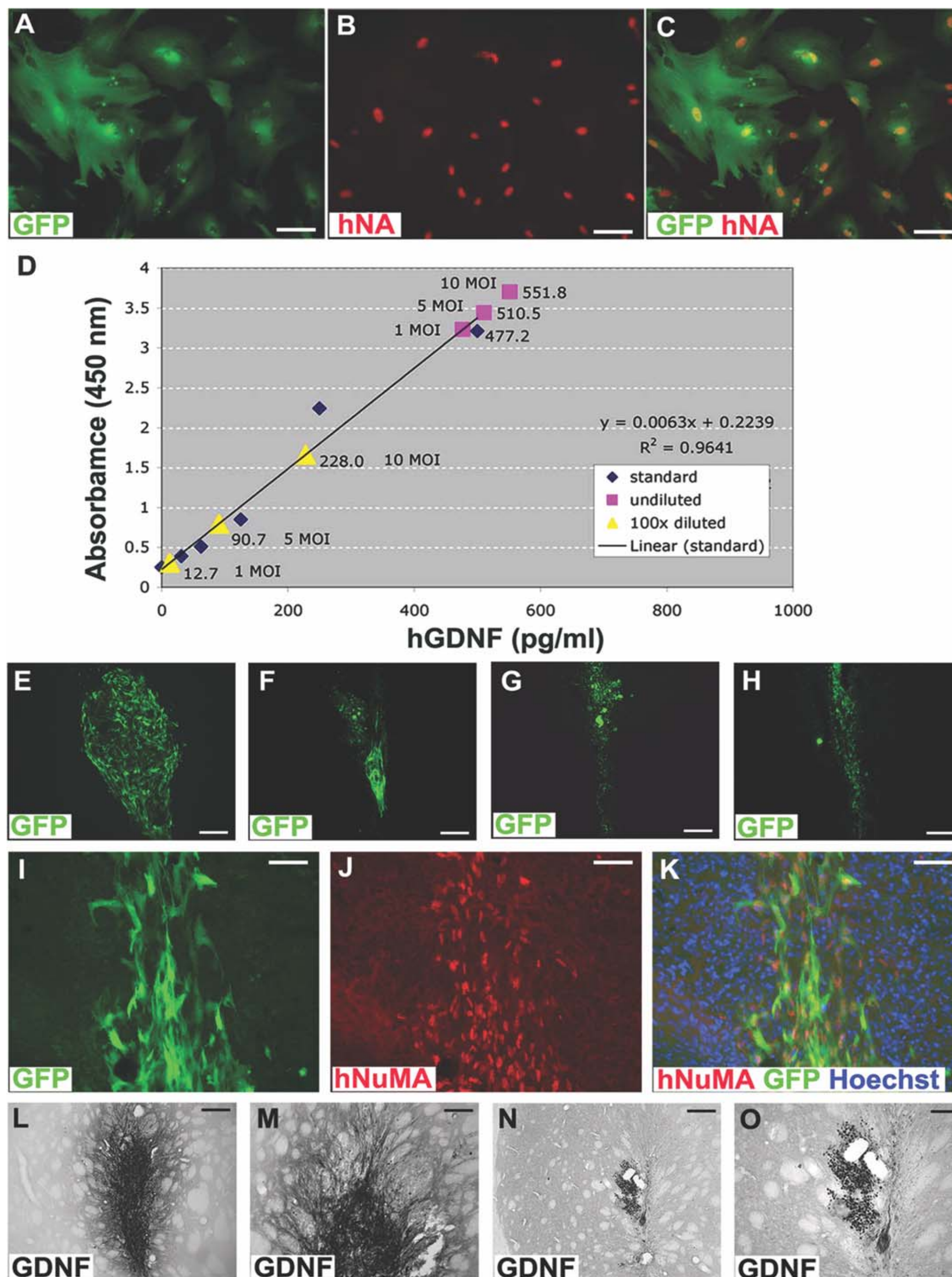

Fig. 1. After transduction with Lenti-CMV-hGDNF-IRES-hrGFP, SB623 cells express GFP and secrete GDNF. **A:** Seventy-two hours after lentiviral transduction, more than 95% of SB623 cells express GFP (green). **B:** In addition, cells were stained with anti-human nuclear antigen (hNA; red) antibody. **C:** Overlaid A and B images. Note that almost all cells display green GFP fluorescence and red hNA staining. **D:** Seventy-two hours after transduction, SB623 cells (yellow + pink) secrete a significant amount of GDNF into the culture medium as determined by ELISA. Undiluted medium samples (pink squares) from cells infected at 1, 5, or 10 MOI and corresponding samples diluted 100-fold (yellow triangles) to be in the linear range of standards (blue diamonds). **E–H:** Adult F344 rats with Lenti-CMV-hGDNF-IRES-hrGFP-transduced SB623 cells grafted into normal striatum at 30,000 cells/μl. Survival of grafted cells at 1, 2, and 4 weeks postgrafting was examined by GFP fluorescence. E: At 1 week postgrafting, numerous GFP-positive cells were detected mostly around the injection site in all rats. F: At 2 weeks postgrafting, a small number of surviving transduced SB623 cells was observed in rats that had received SB623 cells transduced at 5 MOI. G: At 2 weeks postgrafting, an smaller number of surviving cells was detected in rats grafted with SB623 cells transduced at 10 MOI. H: At 4 weeks, only a few cells survived in all grafted rats. **I–K:** At 7 days, grafted SB623 cells transduced with Lenti-CMV-hGDNF-IRES-hrGFP were visualized in striatum by GFP fluorescence and hNuMA-IR. I: Surviving GFP-fluorescent SB623 cells at the graft site. J: The same section as in I stained with hNuMA-IR in red showing surviving cells at 1 week postgrafting; K: Overlay of GFP fluorescence, hNuMA (red), and Hoechst nuclear (blue) staining. Colocalization of hNuMA and Hoechst is shown in pink. **L–O:** Secretion of the GDNF from the grafted transduced SB623 cells is shown with GDNF-IR. L: At 1 week postgrafting, a high density of GDNF-IR cells was observed at the graft site. Also, a GDNF-IR penumbra was observed around grafted SB623 cells, suggesting secretion of GDNF from grafted cells into the surrounding host brain tissue. M: Higher magnification of the graft shown in L. N: At 2 weeks postgrafting, GDNF-IR was confined to the graft site; O: Higher magnification of the graft shown in N. Scale bars = 50 μm in A–C,I–K; 100 μm in E–H,M,O; 200 μm in L,N.

rats. In all rats, a decrease in body weight was observed immediately after surgery and during the first week of the cyclosporine injection. After this initial weight loss, body weight recovered, and the average weight in all treatment groups continued to increase until the end of the experiment. No significant difference in average weight was observed among the treatment groups over the entire experiment (data not shown).

Reduction of Amphetamine-Induced Rotation in Rats Grafted With GDNF-Secreting SB623 Cells

After confirming the ability of SB623 cells to deliver GDNF to the nonlesioned rat striatum, we further tested the ability of GDNF-secreting SB623 cells to reduce behavioral asymmetry in the partial 6-OHDA lesion model of PD. From among 36 rats that exhibited at least six full ipsiversive turns/min at 6 days after lesion (1 day before grafting) and that were assigned to stem cell or control groups, six rats were excluded from the final analysis because of a nosebleed that occurred after amphetamine injection. At 4 weeks postgrafting, rats that had received GDNF-secreting cells (n = 7) had a significantly reduced number of amphetamine-induced rotations compared with control rats ($P < 0.03$; n = 10) and with pregrafting levels ($P < 0.01$; Fig. 2A). Rats grafted with nontransduced (n = 6) or hrGFP-transduced (n = 7) cells had no significant reduction in rotation compared with pregrafting levels or with other groups (Fig. 2A).

In two rats that had received GDNF-secreting SB623 cells, weak GDNF-IR was observed at the graft site (Fig. 2B). In those two rats, the number of amphetamine-induced rotations was reduced by about 50% at 4 weeks postgrafting (from 13 and 14 rot/min before grafting to 7 and 6 rot/min, respectively).

At all time points, preference for ipsilateral forelimb use was not significantly different among the experimental groups. At 4 weeks postgrafting, small levels of improvement were observed in all three stem cell groups; however, these differences were not statistically significant (Supp. Info. Fig. 2).

Rejuvenation of Host DA Fibers Following Grafting of Nontransduced and Transduced SB623 Cells

At 5 weeks postgrafting, no surviving SB623 cells were observed as assessed with GFP fluorescence. This was confirmed by the absence of the hNuMA-IR in all graft sites (Fig. 2C–F). Although there were no surviving cells at 5 weeks postgrafting, numerous rejuvenated host TH-IR fibers were observed in all cellular graft sites (Fig. 2D,F–H), whereas TH-IR fibers were nearly absent in comparable sites in control rats (Fig. 2I). The most prominent rejuvenated host TH-IR fibers were observed in rats that had received GDNF-secreting SB623 cells (Fig. 2G,H), which correlated with the reduction in amphetamine-induced rotation in this experimental group. The effect of grafted SB623 cells on host striatal DA fibers was quantified by measuring the density of TH-IR fibers in the designated area of the grafted side and a corresponding area in the nonlesioned side at two medial and one caudal levels of striatum. The density of TH-IR fibers in the striatum of rats that had received nontransduced and hrGFP-transduced SB623 cells was somewhat higher compared with control rats; however, this did not reach statistical significance (Fig. 2J). In contrast, the density of TH-IR fibers at the graft sites in the medial striatum in rats that had received GDNF-secreting SB623 cells was $\sim$ 3.5-fold higher compared with that of rats that had received hrGFP-transduced SB623 cells (28.5 ± 9.1 vs. 8.1 ± 2.3; $P < 0.05$; Fig. 2J) and $\sim$ 6-fold higher compared with control rats (28.5 ± 9.1 vs. 4.6 ± 0.9; $P < 0.01$; Fig. 2J). This increase was also observed in the caudal striatum (GDNF, 8.6 ± 15.2 vs. control, 7.2 ± 1.4; $P < 0.05$; Fig. 2J). This level was beyond or just at the border of the cell grafts.

DISCUSSION

This study shows that bone marrow-derived SB623 cells can be used as a vehicle to deliver therapeutic levels of GDNF into brain of a rat model of PD. In this model, as originally reported by Sauer and Oertel (1994), the progressive degeneration of DA neurons provides a window of opportunity for the transplanted GDNF-secreting cells to protect and/or to rejuvenate damaged host DA neurons. Numerous studies have shown that rejuvenation and/or regeneration of DA neurons in rodent models of PD can be stimulated by various experimental approaches, including grafts of various tissues, neurotrophic factors, neural progenitors, or stem cells (Bohn et al., 1987, 2000; Bankiewicz et al., 1993, 1994; Bjorklund et al., 1997; Choi-Lundberg et al., 1997; Sayles et al., 2004; Yasuhara et al., 2006; Liu et al., 2007; Shintani et al., 2007; Deierborg et al., 2008; Ebert et al., 2008; Lindvall and Wahlberg, 2008). Moreover, GDNF gene delivery reverses degeneration of DA neurons in aged nonhuman primates (Kordower et al., 2000; Johnston et al., 2009), implying that degeneration of DA neurons in humans afflicted with PD may also be reversed given the appropriate growth environment. Several open-label safety trials using direct intraputamenal infusion of GDNF have reported improvements in the motor and activities of daily living scores in PD patients (Gill et al., 2003; Patel et al., 2005; Slevin et al., 2005). However, double-blind, placebo-controlled clinical studies failed to demonstrate significance of GDNF treatment or caused side effects so that all trials were closed prematurely (Nutt et al., 2003; Lang et al., 2006). The conflicting results from the clinical trials emphasize the need for development of a new methodology for safer and more effective GDNF delivery into the brains of PD patients. To overcome the challenges associated with the complex pharmacokinetics of GDNF protein delivery into the brain, an alternative approach is to use cells genetically modified to secrete this protein in

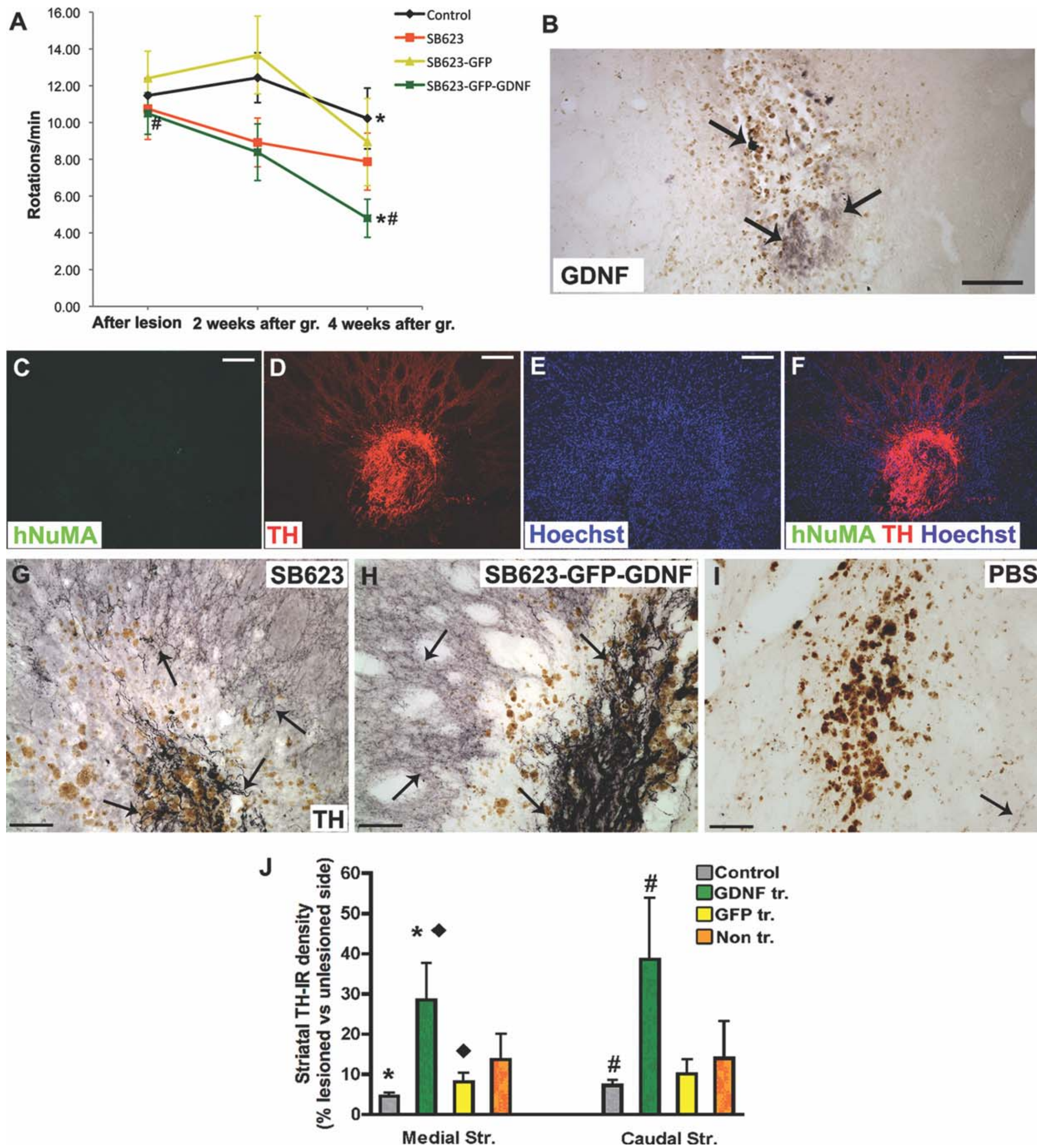

Fig. 2. Effect of nontransduced and GDNF- and/or hrGFP-transduced SB623 cell grafts on recovery of DA fibers in the Fisher 344 rat striatum lesioned with 6-OHDA. Cells were grafted 1 week after the lesion and rats euthanized at 5 weeks postgrafting. **A:** GDNF-transduced SB623 cells reduce amphetamine-induced rotational asymmetry following a 6-OHDA lesion. The majority of rats that received GDNF transduced SB623 cells had significantly fewer rotations at 4 weeks postgrafting compared with postlesion rotations ($^{\#}P < 0.01$) and compared with control rats ($^{*}P < 0.03$). **B:** Expression of GDNF in rat striatum at 5 weeks postgrafting of GDNF-secreting SB623 cells was observed by using nickel-enhanced DAB staining (black). GDNF-IR (arrows) observed in the graft site of a rat that had received GDNF-transduced SB623 cells. **C:** Pseudocolor image showing that there were no hNuMA-IR surviving SB623 cells evident at 5 weeks postgrafting in the striatum of a rat that had received GFP-GDNF-transduced SB623 cells. **D:** TH-IR fibers (red) in the same section shown in C. **E:** Hoechst (blue) nuclear staining in the same section shown in C,D. **F:** Overlay of hNuMA-IR (green), TH-IR (red), and Hoechst nuclear staining (blue). Note the dense network of TH-IR fibers at graft site, but the lack of hNuMA-IR cells, implying a host origin of the TH-IR fibers. **G–I:** Sections stained for TH-IR using nickel-enhanced DAB staining (black). Note dense TH-IR fibers (arrows) in graft sites in a rat that had received nontransduced SB623 cells in G and even denser TH-IR fibers in a rat that had received GFP-GDNF-transduced SB623 cells in H. I: In vehicle-injected control rats, only sparse TH-IR fibers (arrow) were observed around the injection. In B,G–I, brown cells represent macrophages or dead grafted cells in the needle track. **J:** The density of TH-IR fibers in medial and caudal striatum at 5 weeks postgrafting. Values are expressed as percentage of grafted side vs. control (nonlesioned) side. A tendency for higher TH-IR fiber density was detected in rats grafted with nontransduced and hrGFP-transduced SB623 cells than in control rats, whereas a significantly higher density of TH-IR fibers was evident in rats grafted with GDNF-transduced SB623 cells both in medial and in caudal striatum ($^{*}P < 0.01$ vs. corresponding control; $^{\#}P < 0.05$ vs. corresponding control; $^{\blacklozenge}P < 0.05$ vs. rats grafted with hrGFP-transduced SB623 cells). Scale bars = 100 μm in B,C–F; 50 μm in G–I.

a more physiological manner. Ex vivo gene therapy using stem cells or progenitor cells has several advantages compared with protein infusion and in vivo gene therapy, such as the ability to monitor protein production prior to transplantation and production of the protein by engineered donor cells rather than by endogenous cells that might be compromised by disease. In addition, more widespread protein distribution might be achieved as a result of cell migration, and a continuous production of growth factors might occur over an extended period (Imitola et al., 2004; Ebert et al., 2008). Finally, stem cells and progenitors themselves may have effects resulting from secretion of endogenously synthesized neurotrophic factors (Ourednik et al., 2002; Shintani et al., 2007; Glavaski-Joksimovic et al., 2009). In the present study, we used human Notch-induced MSC cells that did not survive past a few weeks in rat brain despite immunosuppression. This limited long-term survival of human Notch-induced MSC cells following transplantation probably was due to the xenografting of human cells into the rat PD model. We believe that syngeneic grafting would result in much better cell survival. In this respect, in ongoing experiments with rat Notch-induced MSC, we have observed numerous surviving cells in 6-OHDA-lesioned rat striatum at 4 weeks postgrafting (unpublished data). Previously, several other cell types have been tested for GDNF delivery in animal PD models, including astrocytes, NSC, and encapsulated GDNF-secreting non-neuronal cells (Tseng et al., 1997; Akerud et al., 2001; Cunningham and Su, 2002; Kishima et al., 2004; Ericson et al., 2005; Behrstock et al., 2006, 2008; Ebert et al., 2008; Lindvall and Wahlberg, 2008). The efficacy of GDNF-secreting bone marrow MSC has been previously shown in 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP) mice (Park et al., 2001) and in a rat model of cerebral ischemia (Horita et al., 2006). In addition, it was recently shown that MSC can directly deliver GDNF into muscle of a rat model of familial amyotrophic lateral sclerosis (ALS; Suzuki et al., 2008) and into contused rat spinal cord (Rooney et al., 2009). These studies with GDNF-secreting SB623 cells are similar to, but distinct from, our study, in which we used SB623 without GDNF transduction. Transplantation of GDNF-secreting bone marrow-derived SB623 cells into the striatum of 6-OHDA partially lesioned rats evoked rejuvenation of host DA fibers around the sites of grafted cells. Moreover, the effect on TH-fiber density extends caudally beyond the location of the grafts into the globus pallidum, implying that GDNF together with other neurotrophic factors secreted from SB623 cells has the ability to affect a larger volume of the striatum. Spared or up-regulated TH-IR fibers have been also observed in the area immediately surrounding transplant sites of GDNF-secreting human neural progenitor cells (Behrstock et al., 2006; Ebert et al., 2008) and GDNF-secreting microspheres (Garbayo et al., 2009). Together these results suggest that GDNF secreted from the genetically modified cells or microspheres is effective in stimulating recovery of damaged DA neurons.

We observed that nontransduced bone marrow-derived SB623 cells also have the capability to rejuvenate host TH-IR fibers in striatum of 6-OHDA partially lesioned rats. This is in agreement with the results of our previous study using micrografts of SB623 cells (Glavaski-Joksimovic et al., 2009). Other investigators have reported therapeutic effects of NICD-transfected bone marrow stromal cells in the 6-OHDA fully lesioned rat PD model (Dezawa et al., 2004) and in a rat model of stroke (Hayase et al., 2009; Yasuhara et al., 2009). Moreover, numerous studies have shown that non-Notch-induced MSC can lead to repair of damaged neuronal tissue (Chopp and Li, 2002; Chen et al., 2003; Prockop, 2003; Aggarwal and Pittenger, 2005; Pisati et al., 2007). Mechanisms proposed to underlie such effects include transdifferentiation of grafted cells, cell fusion, angiogenesis, and modulation of immune cell responses. MSC also secrete neurotrophic factors and other neuroregulatory proteins (Crigler et al., 2006; Pisati et al., 2007; Shintani et al., 2007; Sadan et al., 2009), and such factors may promote recovery of damaged neurons. SB623 cells have also been reported to secrete cytokines and extracellular matrix molecules, although the secretion of GDNF was not detectable (Tate et al., 2008; and Casey Case, unpublished observations). We speculate that the effects of SB623 cells on neurofibers observed in this and previous studies were also achieved through secretion of various trophic factors. Interestingly, in the study of Ebert and colleagues (2008), protection or regeneration of TH-IR fibers in striatum was observed only in rats that had received GDNF-secreting neuroprogenitors and not in those that had received insulin-like growth factor 1 (IGF-1) secreting neuroprogenitors or nontransduced neuroprogenitors. This suggests that brain-derived neuroprogenitors and bone marrow-derived SB623 cells have different capabilities in stimulating repair of DA neurons.

Although the rejuvenated TH-IR fibers were observed in all stem-cell-grafted groups, a statistically significant decrease in the amphetamine-induced rotation was observed only in rats that had received GDNF-transduced SB623 cells. The decrease in amphetamine-induced rotation was observed at 4 weeks postgrafting, although no surviving grafted cells were observed at the end of the experiment. These data suggest that GDNF levels secreted from SB623 cells during the first few days after the grafting were sufficient to exert functional recovery in this rat PD model. At 5 weeks postgrafting, weak GDNF-IR was observed in two rats, which correlated with a prominent reduction in amphetamine-induced rotation. This suggests that, even after the majority of grafted cells had died, there was residual GDNF-IR, although it is not known whether this retained bioactivity. GDNF might also have been taken up by host fibers and retrogradely transported to DA neuronal perikarya in substantia nigra, where it may have acted to rejuvenate these neurons.

In summary, grafts of GDNF-secreting human bone marrow-derived SB623 cells can rejuvenate host

striatal DA fibers and DA-dependent behavioral recovery in a rat model of PD. GDNF-secreting bone marrow-derived SB623 cells may be valuable clinically for PD because of both their intrinsic secretion of neurotrophic factors and their ability to deliver high levels of GDNF to the striatum in an ex vivo gene therapy approach. Another advantage of this MSC approach is that it could be developed as an autologous or allogeneic stem cell therapy for PD. A major challenge for future experiments will be to optimize methods for transplantation of these cells to enhance the distribution and survival of cells as well as to develop methods for regulating GDNF expression.

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