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Practical Induction System for Dopamine-Producing Cells from Bone Marrow Stromal Cells Using Spermine-Pullulan-Mediated Reverse Transfection Method

Kentaro Nagane, M.Eng.,[1,*] Masaaki Kitada, M.D., Ph.D.,[2,3,*] Shohei Wakao, M.H.S.,[2,3] Mari Dezawa, M.D., Ph.D.,[2,3] and Yasuhiko Tabata, Ph.D., D.Med.Sci., D.Pharm.[1]

Introduction of various kinds of exogenous genes is an important step for control of differentiation in stem cell biology and regenerative medicine. However, some kinds of cells are vulnerable to manipulations such as gene delivery. In this context, a gene introduction method with higher efficiency and safety is required. Bone marrow stromal cells (BMSCs) offer possibilities for clinical application because of their potential for expandability and ability to be auto-transplanted. In this study, we established an efficient induction system of dopamine-producing neuronal cells from BMSCs in several species using the spermine-pullulan-mediated reverse transfection technique. In this system, introduced exogenous plasmid genes were successfully transcribed and expressed as proteins in the cytoplasm of BMSCs with the smallest number of cell death. Microtubule-associated protein 2 and anti-beta-tubulin class III$^+$ neurons were successfully delivered from human, monkey, and mouse BMSCs, and further treatment with trophic factors promoted differentiation of induced neuronal cells into dopamine-producing cells that were positive for tyrosine hydroxylase and secreted dopamine after high K^+ stimulation in high-performance liquid chromatography analysis. Our study indicates the availability of the reverse transfection method for the induction of dopamine-producing neuronal cells from BMSCs, which is expected to apply to cell-based therapy in Parkinson's disease.

Introduction

An efficient method of introducing genes into target cells is necessary not only for basic research, but also for practical applications. Currently, lipofection and electroporation are the most widely used methods for gene delivery with the recent improvement of their introduction efficiency and of cell damage. Particularly, introduction of various kinds of exogenous genes is occasionally an important step for the control of differentiation and induction of stem cells. However, because some kinds of cells are vulnerable to lipofection or electroporation, cell damage during the induction procedure is for a barriar to actualization of cell-based therapy. Furthermore, gene introduction with higher efficiency and safety is also needed for practical use.

Bone marrow contains mesenchymal cells, also called bone marrow stromal stem cells or bone marrow stromal cells (BMSCs) that possess the potential to differentiate into other cell types.[1–11] Human BMSCs have a particularly high proliferation ability (20–100 mL of bone marrow aspirate provides 1×10^7 BMSCs within several weeks).[12] BMSCs are thus promising candidates for clinical application because they are easily isolated from patient or donor bone marrow aspirates and are readily expanded *in vitro* for auto- or allo-transplantation without posing major ethical problems.

BMSCs have been reported to differentiate into mesenchymal lineage cells such as osteocytes and chondrocytes, and these systems have already been applied for clinical treatment such as for osteoarthritis.[7,13] Recently, we established a method of systematically and specifically inducing neuronal cells and muscle cells from BMSCs with a view to pioneering auto- and allo-cell transplantation therapy in neuro- and muscle degenerative diseases.[14–16] We have reported that highly efficient and specific induction of post-mitotic functional neuronal cells, without glial differentiation, can be achieved using gene transfer of Notch intracellular domain (NICD) followed by the administration of a certain combination of trophic factors. When glial cell line–derived

[1]Department of Biomaterials, Field of Tissue Engineering, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan.
[2]Department of Anatomy and Neurobiology, Kyoto University Graduate School of Medicine, Kyoto, Japan.
[3]Department of Stem Cell Biology and Histology, Tohoku University Graduate School of Medicine, Aoba-ku, Sendai, Japan.
*These authors contributed equally to this work.

neurotrophic factor (GDNF), known to promote the generation and development of midbrain dopaminergic neurons,[17] was administered to the induced neuronal cells, the cells further differentiated into dopamine-producing cells, which release dopamine into the culture medium in response to high K^+ depolarizing stimuli. Transplantation of these cells into striatum of rat models of Parkinson's disease showed significant recovery in pharmacological and non-pharmacological behavior tests. Furthermore, most of the transplanted cells integrated into the host striatum as dopamine transporter—and tyrosine hydroxylase (TH)-positive cells. Transplantation of human-induced dopaminergic neurons into the striatum of Parkinson's model rats under the control of immunosuppressanta also showed significant improvement on a behavior test in rat models.[15]

Although this method provides a basis for auto-cell-transplantation therapy, which is expected to be one of the effective treatments for Parkinson's disease, several matters need to be resolved before they can be used clinically. The strategy for delivering the NICD gene into BMSCs with safety, high efficiency, and low cytotoxicity is the most important primary concern. Because tentative expression of the NICD gene in BMSCs is the key point for the induction of dopaminergic neurons and because their differentiation into post-mitotic neurons was inhibited when NICD was stably expressed in BMSCs using retrovirus or lentivirus vectors, plasmid transfection that ensures tentative expression of the introduced gene is considered suitable for the systemic induction of dopaminergic neurons from BMSCs.[15]

In past studies, we have used lipofection for the introduction of the NICD plasmid gene into BMSCs[14–16] because lipofection is one of the most general methods for plasmid gene transfection. Despite its usefulness, its cytotoxicity is often questioned in human BMSCs and the ratio of NICD introduction is approximately 30% to 40% at best. Moreover, for preclinical study, safety and efficiency need to be verified in higher mammals such as monkeys, although monkey BMSCs are vulnerable to any kind of stress or stimulation. Because most monkey BMSCs die in the process of lipofection and after selection, preclinical study in monkeys was found to be difficult to accomplish. Furthermore, gene delivery using virus-mediated gene transfer has been applied to BMSCs to modify their function or to control their differentiation.[18,19] However, the gene delivery method, other than mediating any viruses with high efficiency and low cytotoxicity, would be preferable for practical use.

Gene transfer methods fall largely into two categories: viral and non-viral transfection techniques. The former has been used extensively in biological and medical researches because of its high efficiency of gene transfection. However, because the viral technique is practically limited from a clinical viewpoint, several non-viral carriers have been explored to improve transfection efficiency and cytotoxicity for clinical applications. For this purpose, we have developed the reverse transfection method,[20] in which cells are cultured on the plasmid DNA-carrier complex that achieves high cellular internalization of plasmid DNA due to direct and continuous contact with the cell membrane. Reverse transfection can be performed in the presence of serum, which gives cells better conditions to increase their viability in culture.[20]

We applied this reverse transfection method for the introduction of the NICD gene first for monkey BMSCs, to which gene transfer by lipofection is virtually impossible. Gene delivery using this method was successful, with high efficiency and low cytotoxicity so as to induce dopaminergic neurons. These availabilities were also confirmed in human and mouse BMSCs.

BMSCs offer possibilities for clinical application, because they can be efficiently expanded *in vitro* to acquire a therapeutic scale with fewer ethical problems. In addition, bone marrow transplantation has already been widely performed. Based on these advantages, establishment of a stable induction system of dopaminergic neurons from BMSCs will contribute to the promotion of cell-based therapy in Parkinson's disease. Our study indicates the availability of the reverse transfection method for this system.

Materials and Methods

Materials

Pullulan, with a weight-average molecular weight of 47,300, and spermine were purchased from Hayashibara Biochemical Laboratories (Okayama, Japan) and Sigma (St. Louis, MO), respectively. Other chemical agents were purchased from Nacalai (Kyoto, Japan) and were used without further purification, unless otherwise indicated.

Preparation of cationized pullulan derivative

Spermine was introduced to the hydroxyl groups of pullulan using a N, N′-carbonyldiimidazole (CDI) activation method.[21] Dehydrated dimethyl sulfoxide containing 50 mg of pullulan (50 mL) was supplemented with 1.87×10^3 mg of spermine and 2.25×10^2 mg of CDI. After agitation at 35°C for 20 h, the reaction mixture was dialyzed with ultra-pure double-distilled water (DDW) for 2 days through a dialysis membrane (cut-off molecular weight: 12,000–14,000; Viskase, Willowbrook, IL). The dialyzed solution was freeze-dried to obtain spermine-introduced pullulan (spermine-pullulan). When measured using conventional elemental analysis, the molar extent of spermine introduced into the hydroxyl groups of pullulan was confirmed to be 14.5 mol% by volume.

Plasmids

The mouse NICD (constitutive active form) complementary DNA, coded for a transmembrane region that included a small fragment of extracellular domain and followed by a sequence encoding the entire intracellular domain of mouse Notch1 (initiating at amino acid 1,703 and terminating at the 3′ untranslated sequence),[22] was introduced into BMSCs.[15] This fragment was sub-cloned into a pCI-neo vector (Promega, Madison, WI) (pCI-NICD). To evaluate the expression efficiency, pCI-NICD green fluorescent protein (GFP) was also constructed by inserting an enhanced GFP gene derived from a permuted enhanced GFP (pEGFP)-N2 vector (Clontech, Palo Alto, CA). To evaluate the intracellular localization of the transfected plasmid DNA, Label IT® NUCLEIC ACID LABELING KITS (Mirus, Madison, WI) was used for Cy5 labeling of the pCI-NICD plasmid DNA for spermine-pullulan-mediated reverse transfection. For luciferase assay, pGL3 vector (Promega) coding for a firefly luciferase gene was used.

Preparation and culture of BMSCs

The usage of human BMSCs in this study was approved by Ethics Committee of Tohoku University and

Kyoto University Graduate School and Faculty of Medicine. All animal experiments were approved by the Animal Care and Experimentation Committee of Tohoku University and Kyoto University Graduate School of Medicine.

BMSCs from monkey, mouse, and human were used in this study. Bone marrow aspirates were obtained from pelvic bone of Macaca fascicularis and subjected to primary culture to establish monkey BMSCs (monBMSCs). Mouse BMSCs were isolated from the femoral bone of 8-week-old male C57BL/6J and cultured as described in our previous report.[23] Human BMSCs were purchased from Cambrex (East Rutherford, NJ) and SanBio (San Francisco, CA). BMSCs were maintained in alpha-minimum essential medium (α-MEM) (Sigma) containing 10% fetal calf serum (FCS) and kanamycin at 37°C with 5% carbon dioxide (CO_2).

Preparation of polyion complexes

Polyion complex (PIC) was prepared by mixing an aqueous solution of spermine-pullulan with the plasmid DNA diluted in phosphate buffered saline solution (PBS, 10 mM, pH 7.4) followed by incubation for 15 min at room temperature. The PIC composition was calculated based on the nitrogen number of the spermine-pullulan (N) per the phosphorus number of plasmid DNA (P) (the N/P ratio). The apparent molecular size of PIC was measured using DLS (DPA-60HD instrument, Otsuka Electronic, Osaka, Japan) equipped with an Ar^+ laser at a detection angle of 90° at 25°C for 15 min. In the present study, the autocorrelation function of samples was analyzed based on the cumulative method so that the computer software calculated the Rs value automatically and expressed it as the apparent molecular size. The zeta potential of PIC was measured using electrophoretic light scattering (DPA-60HD).

Reverse transfection method

The reverse transfection method was performed as described in our previous report.[20] Briefly, 90.1 mg of succinic anhydride was added to 20 mL of 100 mg/mL gelatin solution in dimethyl sulfoxide, followed by agitation at room temperature for 18 h to allow the introduction of the carboxyl groups to the amino groups of gelatin for anionization. Judging from the extent of amino group decrementation determined by the trinitrobenzene sulfonic acid method,[24] the molar amount of introduced carboxylic groups was revealed to be 100 mol% by volume.

The aqueous solution of the anionized gelatin (100 mg/mL) and ProNectin (200 mg/mL) was applied to culture dishes, followed by incubation at 37°C for 2 h to allow adhesion of these two reagents to the surface of the culture dishes. After being washed with PBS, plasmid DNA-spermine-pullulan complexes were dispersed as 0.75 μg of the plasmid DNA per cm^2 onto culture dishes coated with gelatin and ProNectin. After 1 h incubation, the solution was removed, and BMSCs at the fourth subculture were re-plated onto the complex-coated culture dishes. Cy5-labeled plasmid DNA was used to visualize the state of fixation.

Cell viability assay

Cytotoxicity was evaluated using a cell counting kit (Nacalai) according to our previous study with slight modification.[20] Briefly, monBMSCs were subjected to the transfection with pCI-NICD plasmid DNA by using either of the following methods; i.e., lipofectamine2000 (with 2.0 mg of the pCI- NICD plasmid DNA), or the reverse transfection of pCI-NICD plasmid DNA (2.0 mg)-spermine-pullulan complexes. For lipofection, cells were incubated with the lipofectamine2000 for 4hr, washed out several times and then subjected to the assay after 3 days. For reverse transfection, cells were left in the pCI-NICD plasmid DNA (2.0 mg)-spermine-pullulan complexes medium for 3 days and subjected directly to the assay. For cell viability assay, the medium was changed to α-MEM with FCS, and 100 μL of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2.4-disulfophenyl)-2H-tetrazolium (WST-8) solution was added and the cells incubated for another 3 h. The absorbance of samples was measured at 450 nm using a VERSAmax microplate reader (Molecular Devices, Sunnyvale, CA). The percentage of cell viability was expressed as 100% for non-transfected control cells.

Reporter gene assay

After transfection of pGL3, cells were washed twice with PBS, lysed in 100 μL of cell culture lysis reagent (Promega), and transferred into a micro reaction tube, and the cell debris was separated using centrifugation (14,000 rpm, 20 min). Then 100 μL of luciferase assay reagent (Promega) was added to 20 μL of supernatant while the relative light unit of the sample was determined using a luminometer (Micro-LumatPlus LB 96 V, Berthold, Tokyo, Japan). The total protein of each well was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL) according to the manufacturers' instructions. The relative light unit was divided by the protein amount to normalize the influence of number variance of cells on luciferase activity. Reporter gene assay was independently repeated at least for 3 times in each group. As a control, Lipofectamine 2000 reagent was used to

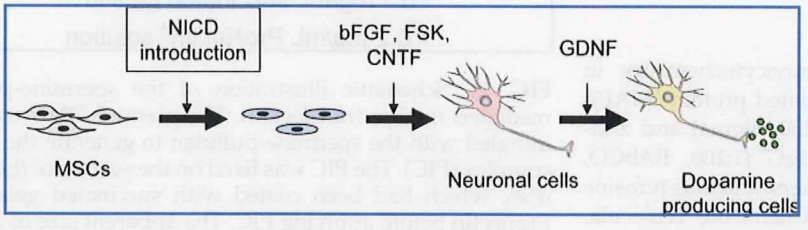

FIG. 1. Induction system of dopaminergic neurons. Bone marrow stromal cells (BMSCs) were transfected with the pCI-Notch intracellular domain (NICD) plasmid, selected using G418. Treatment with trophic factors basic fibroblast growth factor (bFGF), forskolin (FSK), and ciliary neurotrophic factor (CNTF) leads BMSCs to differentiation into neuronal cells. Further treatment of cells with glial cell line–derived neurotrophic factor induces dopaminergic neurons. Color images available online at www.liebertonline.com/ten.

transfect 0.4 μg of plasmid per cm^2 for 4 h with 10% FCS, which was a milder condition the supplier's recommendation. Cytotoxicity was evaluated using a cell counting kit (Nacalai) according to the manufacturer's recommendation.

Induction of dopamine-producing neurons

The induction procedure is summarized in Figure 1. BMSCs were transfected with pCI-NICD plasmid using the spermine-pullulan-mediated reverse transfection method as described above and followed by G418 selection for 5 to 7 days. For the induction of post-mitotic mature neuronal cells, pCI–NICD-introduced BMSCs were re-plated at a cell density of 2,080 cells/cm^2, and trophic factors (5 μM of forskolin (FSK; Calbiochem, La Jolla, CA), 10 ng/mL of basic fibroblast growth factor (bFGF; Peprotech, London, UK), and 10 ng/mL of ciliary neurotrophic factor (CNTF; R&D Systems, Minneapolis, MN) in α-MEM containing 5% FCS were supplied as described previously.[15] For induction of dopamine-producing cells, GDNF (50 ng/mL; Peprotech) was further supplied to the trophic factor–treated cells.[15] Two kinds of controls, naïve monBMSCs and monBMSCs treated with three trophic factors (bFGF, FSK, and CNTF) followed by GDNF incubation without the spermine-pullulan-mediated reverse transfection of pCI-NICD plasmid (TF-monBMSCs), were prepared for immunocytochemistry and dopamine release assay.

Dopamine release assay

Dopamine release assay was performed according to a previous study.[25] Briefly, cells were washed in a low K^+ solution (20 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 140 mM of sodium chloride (NaCl), 4.7 mM of potassium chloride (KCl), 2.5 mM of calcium chloride, 1.2 mM of magnesium sulphate, 1.2 mM of potassium dihydrogen phosphate, and 11 mM of glucose, pH 7.4) and incubated in the low-K^+ solution for 5 minutes, and then the medium was replaced with a high-K^+ solution (same as the low-K^+ solution except for 85 mM of NaCl and 60 mM of KCl) for 5 min. Concentration of dopamine was determined using high-performance liquid chromatography (HPLC) using a reverse-phase column and an electrochemical detector system (Eicom, Kyoto, Japan). The mobile phase was composed of 0.1 M of phosphate buffer (pH 6.0), 20% methanol, 500 mg/L of octanesulfonic acid, and 50 mg/L of disodium ethylene diamine tetraacetic acid. The injection volume was 20 μL, and the working electrode was compared with a silver/silver chloride reference electrode and set at 0.45 V.[25] The amount of dopamine release was measured according to cell number, which was counted after trypsinization.

Immunocytochemistry

Primary antibodies used for immunocytochemistry in this study were anti-microtubule associated protein (MAP)-2ab mouse immunoglobulin (Ig)G (1:250, Sigma) and anti-beta-tubulin class III (Tuj-1) mouse IgG (1:100, BAbCO, Richmond, CA) as neuron-specific markers, and anti-tyrosine hydroxylase (TH) rabbit IgG (1:1,000, Chemicon, Temecula, CA) as a marker for dopaminergic neurons. Secondary antibodies were anti-mouse or anti-rabbit IgG antibodies conjugated to Alexa488 (Molecular Probes, Invitrogen). Nuclei were counter-stained with 4′, 6-diamidino-2-phenylindole and inspected using confocal laser microscopy (Nikon Corporation, Tokyo, Japan).

Statistical analysis

Values were expressed as the mean ± the standard deviation of the mean. Crude data were analyzed using two-way analysis of variance, and then differences between means were further analyzed using the Fisher's protected least

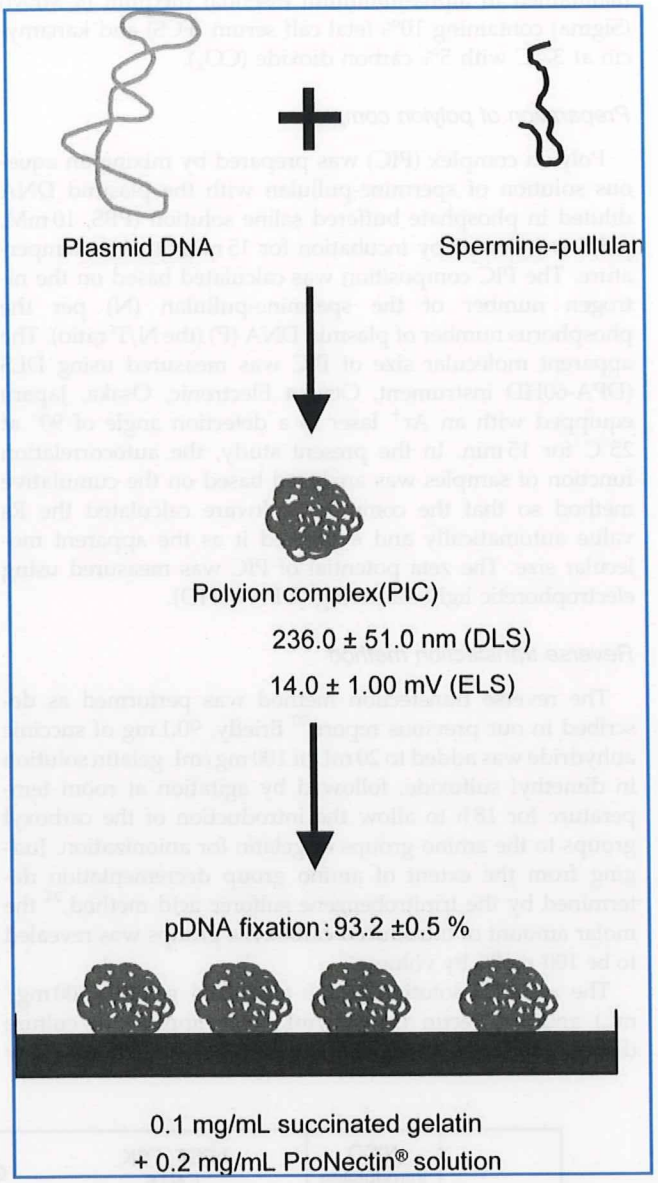

FIG. 2. Schematic illustration of the spermine-pullulan-mediated reverse transfection. The plasmid DNA was intermingled with the spermine-pullulan to generate the polyion complex (PIC). The PIC was fixed on the surface of the culture dish, which had been coated with succinated gelatin and Pronectin before applying PIC. The apparent size of PIC was 236.0 ± 51.0 nm when measured using dynamic light scattering (DLS). The zeta potential of PIC was 14.0 ± 1.00 mV when measured using electrophoretic light scattering (ELS).

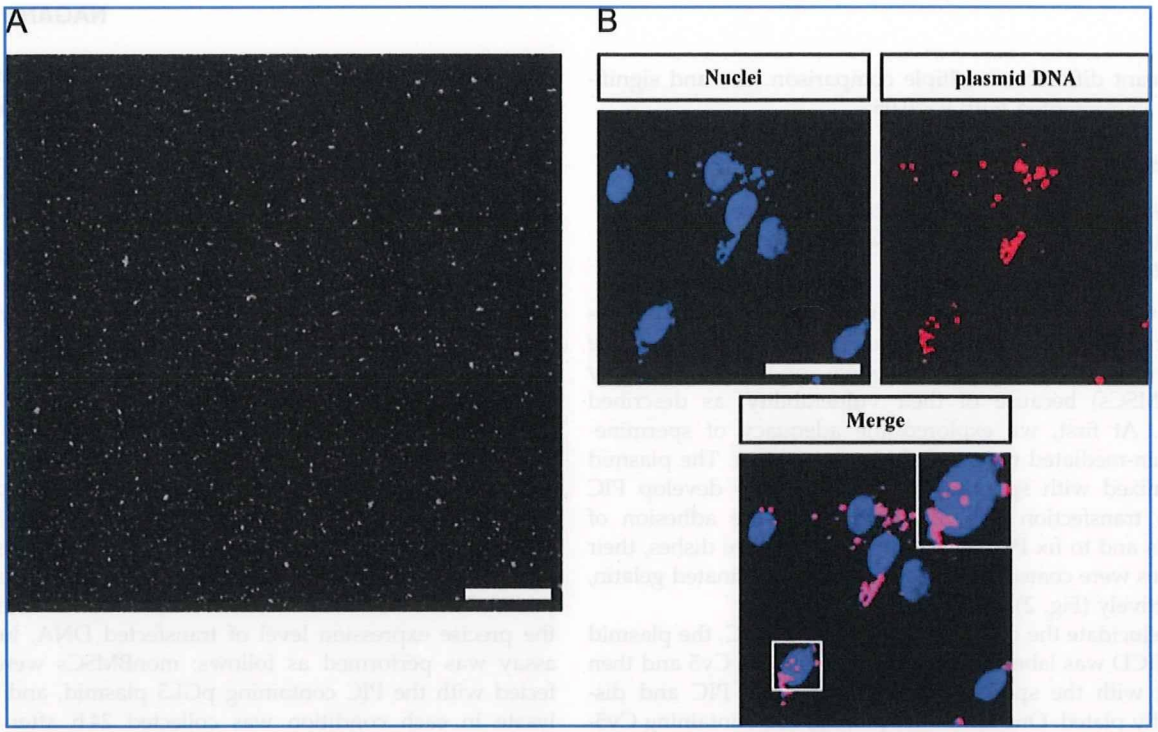

FIG. 3. (**A**) Confocal laser microscopic image of the surface of culture dishes fixed with the polyion complex (PIC) containing Cy5-labeled plasmid DNA. Plasmid DNA was visualized using Cy5 fluorescence. (**B**) Confocal laser microscopic images of monkey bone marrow stromal cells transfected with the Cy5-labeled plasmid DNA (red) using the spermine-pullulan-mediated reverse transfection technique. The Cy5-labeled plasmid DNA could be found as punctuate signals in cytoplasm and the nucleus 24 h after starting the reverse transfection. Nuclei were counterstained using 4′, 6-diamidino-2-phenylindole (blue). Scale bars: 200 μm (**A**) and 100 μm (**B**). RLU, relative light unit.

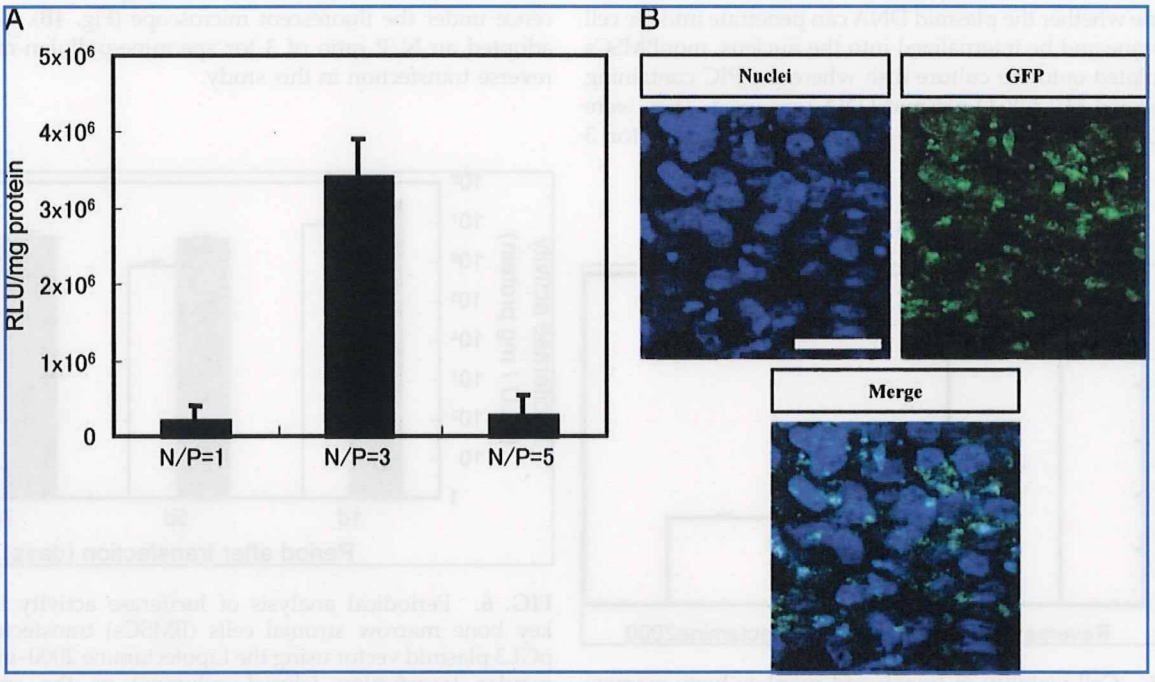

FIG. 4. (**A**) Luciferase assay for the determination of N/P ratio. Three conditions for the nitrogen number of the spermine-pullulan (N) per the phosphorus number of plasmid DNA (P) (the N/P ratio), 1, 3, and 5, were employed for the spermine-pullulan-mediated reverse transfection with pGL3 vector. Substantial activity of luciferase was observed in the N/P ratio condition 3–more than 10 times as great as that of 1 or 5. (**B**) Green fluorescent protein (GFP) expression in monkey bone marrow stromal cells (monMSCs) transfected with pCI-Notch intracellular domain (NICD) GFP 3 days after starting the spermine-pullulan-mediated reverse transfection in the N/P ratio of 3. GFP fluorescence could be detected in monMSCs transfected with pCI-NICD GFP but not in those without transfection (not shown). 4′, 6-diamidino-2-phenylindole was used for counterstaining of nuclei (blue). Scale bar: 100 μm. RLU, relative light unit; GFP, green fluorescence protein.

significant difference multiple comparison test, and significance was accepted with $p < 0.05$.

Results

Intracellular localization of the transfected gene introduced using spermine-pullulan-mediated transfection

To test the effectiveness of the spermine-pullulan-mediated reverse transfection system, *Macaca fascicularis* bone marrow was used as the source of BMSCs (namely monBMSCs) because of their vulnerability, as described above. At first, we explored the adequacy of spermine-pullulan-mediated reverse transfection system. The plasmid was mixed with spermine-pullulan so as to develop PIC before transfection (Fig. 2). To enhance the adhesion of BMSCs and to fix PIC on the surface of culture dishes, their surfaces were coated with ProNectin and succinated gelatin, respectively (Fig. 2).

To elucidate the distribution of the fixed PIC, the plasmid pCI-NICD was labeled with a fluorescent dye Cy5 and then mixed with the spermine-pullulan to make PIC and dispersedly plated. One hour after plating, PIC containing Cy5-labeled pCI-NICD plasmid DNA was found successfully fixed on the surface of ProNectin-coated culture plates after stringent washing (Fig. 3A).

There are five key steps for the expression of plasmid DNA: (1) attachment of plasmid DNA onto the cell surface, (2) internalization of the plasmid DNA into cytosol, (3) endosomal escape of the plasmid DNA, and (4 and 5) transfer and internalization of the plasmid DNA into the nucleus. To examine whether the plasmid DNA can penetrate into the cell membrane and be internalized into the nucleus, monBMSCs were plated onto the culture dish where the PIC containing Cy5-labeled pCI-NICD plasmid DNA was fixed. They were then incubated with α-MEM containing 10% serum for 3 days at 37°C, 5% CO_2 for the transfection of DNA. After reverse transfection, monBMSCs were fixed, and intracellular localization of PIC was examined according to the detection of Cy5 fluorescence-labeled plasmid DNA. Punctate distribution of internalized plasmid DNA was recognized in the cytoplasm, and some of them were detected in the nucleus as well (Fig. 3B). These findings demonstrate that the plasmid DNA was successfully introduced into the cytosol and nucleus, showing that the transcription of transfected DNA took place within the transfected BMSCs.

Optimization of the ratio of spermine-pullulan to plasmid DNA

To optimize the volume ratio of the spermine-pullulan and the plasmid DNA for subfection, the optimal ratio of the nitrogen number of the spermine-pullulan to the phosphorus number of the plasmid DNA (the N/P ratio) was determined. Three N/P ratios (1, 3, and 5) were set. To evaluate the precise expression level of transfected DNA, luciferase assay was performed as follows; monBMSCs were transfected with the PIC containing pGL3 plasmid, and the cell lysate in each condition was collected 24 h after reverse transfection. Luciferase assay demonstrated that an N/P ratio of 3 gave more than 10 times more luciferase activity than a ratio of 1 or 5, indicating that the best N/P ratio is 3 for functional protein expression in spermine-pullulan-mediated reverse transfection (Fig. 4A).

Under this reverse transfection condition, the gene product of plasmid encoding pCI-NICD-GFP in transfected monBMSCs could be detected according to its GFP fluorescence under the fluorescent microscope (Fig. 4B). Thus, we adopted an N/P ratio of 3 for spermine-pullulan-mediated reverse transfection in this study.

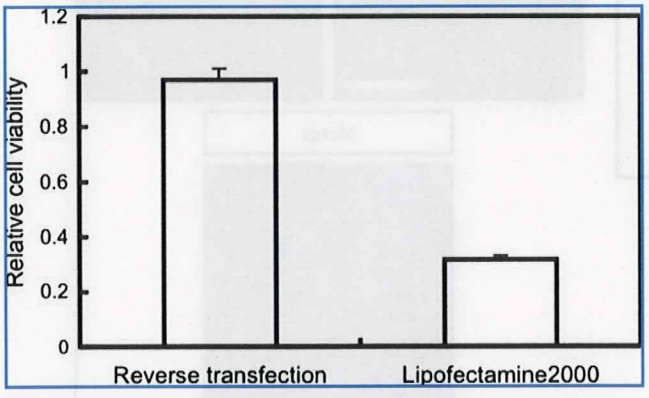

FIG. 5. Cell viability of transfected monkey bone marrow stromal cells (BMSCs) in which the spermine-pullulan-mediated reverse transfection (left columns) or Lipofectamine 2000–mediated transfection (right columns) delivered the pCI-Notch intracellular domain (NICD) plasmid 3 days before the evaluation. MSCs transfected using the spermine-pullulan-mediated reverse transfection method exhibited higher cell viability (97.0 ± 4. 1%) than that of Lipofectamine 2000 (31.6 ± 1.4%).

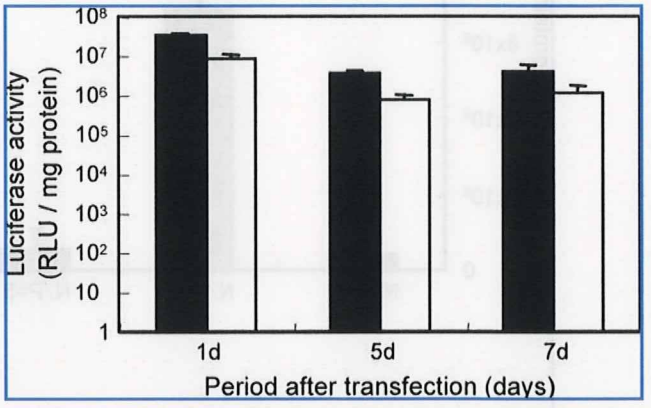

FIG. 6. Periodical analysis of luciferase activity in monkey bone marrow stromal cells (BMSCs) transfected with pGL3 plasmid vector using the Lipofectamine 2000–mediated regular transfection (closed columns) or the spermine-pullulan-mediated reverse transfection technique (open columns) 1, 5, and 7 days after transfection. Measured right units were normalized to be analyzed using two-way analysis of variance and Fisher's protected least significant difference multiple comparison test. No significant difference could be found in luciferase activity between the regular transfection and reverse transfection in each period. RLU, relative light unit.

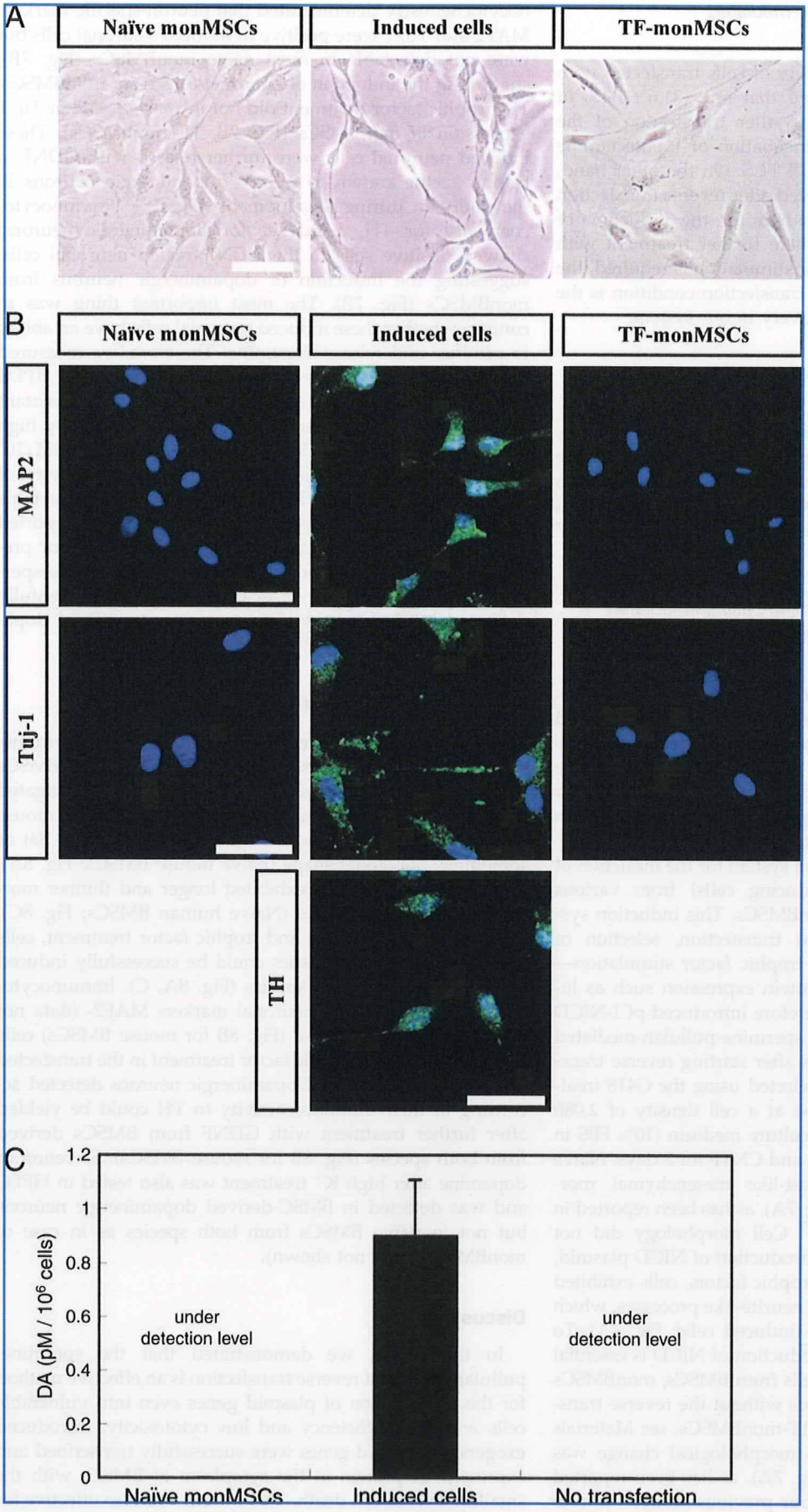

FIG. 7. Morphological and functional characterization of naïve monkey bone marrow stromal cells (monBMSCs) induced neuronal cells and trophic factor–treated monMSCs without the reverse transfection of pCI-Notch intracellular domain (NICD; TF-monMSCs, see text). (**A**) Phase contrast images. Induced neuronal cells exhibited round cell bodies with neurite-like processes. (**B**) Immunocytochemistry for neuronal markers. Induced neuronal cells were positive for neuron-specific antigens microtubule-associated protein (MAP)2 and anti-beta-tubulin class III (Tuj-1). Tyrosine hydroxylase–positive neuronal cells detected after further treatment of induced neuronal cells with glial cell line–derived neurotrophic factor. Most naïve monMSCs and TF-monMSCs were negative to MAP2 and Tuj-1. (**C**) Dopamine release assay detected using high-performance liquid chromatography. Sequential treatment of low and high K^+ condition stimulated the secretion of dopamine. A substantial increase in dopamine secretion was detected only in induced neuronal cells (middle column) but was under detection levels in naïve monMSCs and TF-monMSCs (left and right columns, respectively). Data were collected from three independent experiments. Scale bars: 100 μm.

Low toxicity of spermine-pullulan-mediated transfection

Next, we measured the viability of cells transfected with plasmid DNA. Figure 5 showed that only 31.6 ± 1.4% of transfected monBMSCs survived after transfection of the pCI-NICD plasmid using 4-h incubation of Lipofectamine 2000 even in the presence of 10% FCS. On the other hand, 97.0 ± 4.1% of monBMSCs survived after reverse transfection at 3 days. Considering our method for the induction of neuronal cells from BMSCs, where further treatment with G418 selection and trophic factor stimulation is required, the low cytotoxicity of this reverse transfection condition is the great advantage of the gene delivery in our system.

Efficiency of spermine-pullulan-mediated transfection

To test the efficiency of this transfection system, the sequential luciferase assay was performed from day 1 to 7 after starting the reverse transfection. Comparing the luciferase activity of spermine-pullulan-mediated reverse transfection with that of Lipofectamine 2000, there was no statistical difference in their protein expression level from day 1 to 7 (Fig. 6).

Functional assessment of spermine-pullulan-mediated transfection through neuronal cell induction assay from BMSCs

We have estimated the viability and efficiency of this spermine-pullulan-mediated reverse transfection system and found that the viability was much higher than, and the efficiency was as same as, that of Lipofectamine 2000. The efficiency was estimated according to luciferase activity—a functional but simple assay for protein expression. The main objective in this study was to focus on verifying the availability of this reverse transfection system for the induction of neuronal cells (dopamine-producing cells) from various kinds of BMSCs, including monBMSCs. This induction system comprised NICD plasmid transfection, selection of transfected cells by G418, and trophic factor stimulation—much different from simple protein expression such as luciferase activity (Fig. 1). We therefore introduced pCI-NICD plasmid into monBMSCs using spermine-pullulan-mediated reverse transfection. Three days after starting reverse transfection, transfected cells were selected using the G418 treatment for 5 to 7 days, re-plated at a cell density of 2,080 cells/cm^2, and incubated with culture medium (10% FBS in α-MEM) containing bFGF, FSK, and CNTF for 3 days. Naïve monBMSCs exhibited fibroblast-like mesenchymal morphology (naïve monBMSCs; Fig. 7A), as has been reported in BMSCs from other species.[26,27] Cell morphology did not change significantly after the introduction of NICD plasmid, although after treatment with trophic factors, cells exhibited round cell bodies and extended neurite-like processes, which were similar to neuronal cells (induced cells; Fig. 7A). To evaluate whether the gene introduction of NICD is essential for the induction of neuronal cells from BMSCs, monBMSCs treated with three trophic factors without the reverse transfection of pCI-NICD plasmid (TF-monBMSCs, see Materials and Methods) were used. No morphological change was observed in TF-monBMSCs (Fig. 7A), as has been reported using rat and human cells in our previous study.[15] Immunocytochemistry demonstrated that neuron-specific markers MAP2 and Tuj-1 were positive in induced neuronal cells but were not detectable in most naïve monBMSCs (Fig. 7B), suggesting the induction of neuronal cells from monBMSCs. The trophic factor treatment did not increase MAP2 or Tuj-1 expression in monBMSCs (Fig. 7B, TF-monBMSCs). These induced neuronal cells were further treated with GDNF, a trophic factor known to induce dopaminergic neurons in the midbrain during development (Fig. 1).[17] Immunocytochemistry for TH, a marker for dopaminergic neurons, showed positive cells in the GDNF-treated neuronal cells, suggesting the induction of dopaminergic neurons from monBMSCs (Fig. 7B). The most important thing was to confirm whether these induced neuronal cells have an ability to produce and release dopamine. Therefore, we measured the secretion of dopamine by these induced cells using HPLC (Fig. 7C). The dopamine activity in the culture supernatant was measured after induction of dopamine release by high K^+ depolarizing stimuli; induced cells marked 0.906 pM/10^6 cells of dopamine release, whereas dopamine activity of naïve monBMSCs and TF-monBMSCs were undetectable (Fig. 7C). These results were consistent with those reported in rat BMSC-derived dopamine-producing cells in our previous report.[15] These findings demonstrated that the spermine-pullulan-mediated reverse transfection successfully delivered NICD plasmid so as to induce functional dopamine-producing neuronal cells from monBMSCs.

Neuronal induction in BMSCs from other species

The above data suggest the advantage of a spermine-pullulan-mediated reverse transfection system for BMSCs from other animal species as well. We therefore investigated BMSCs from mice and humans. The morphology of mouse naïve BMSCs is similar to that of monkeys; they are flat or sometimes polygonal shape (naïve mouse BMSCs; Fig. 8A), whereas human BMSCs exhibited longer and thinner morphology than monBMSCs (Naïve human BMSCs; Fig. 8C). After NICD introduction and trophic factor treatment, cells with neuronal characteristics could be successfully induced from BMSCs from both species (Fig. 8A, C). Immunocytochemistry showed that neuronal markers MAP2- (data not shown) and Tuj-1-positive (Fig. 8B for mouse BMSCs) cells were detected after trophic factor treatment in the transfected BMSCs in each species. Dopaminergic neurons detected according to their immunoreactivity to TH could be yielded after further treatment with GDNF from BMSCs derived from both species (Fig. 8B for mouse BMSCs). Secretion of dopamine after high K^+ treatment was also tested in HPLC and was detected in BMSC-derived dopaminergic neurons but not in naïve BMSCs from both species as in case of monBMSCs (data not shown).

Discussion

In this study, we demonstrated that the spermine-pullulan-mediated reverse transfection is an effective method for the introduction of plasmid genes even into vulnerable cells with high efficiency and low cytotoxicity. Introduced exogenous plasmid genes were successfully transcribed and expressed as protein in the cytoplasm of BMSCs with the smallest level of cell death. This system was also effective for

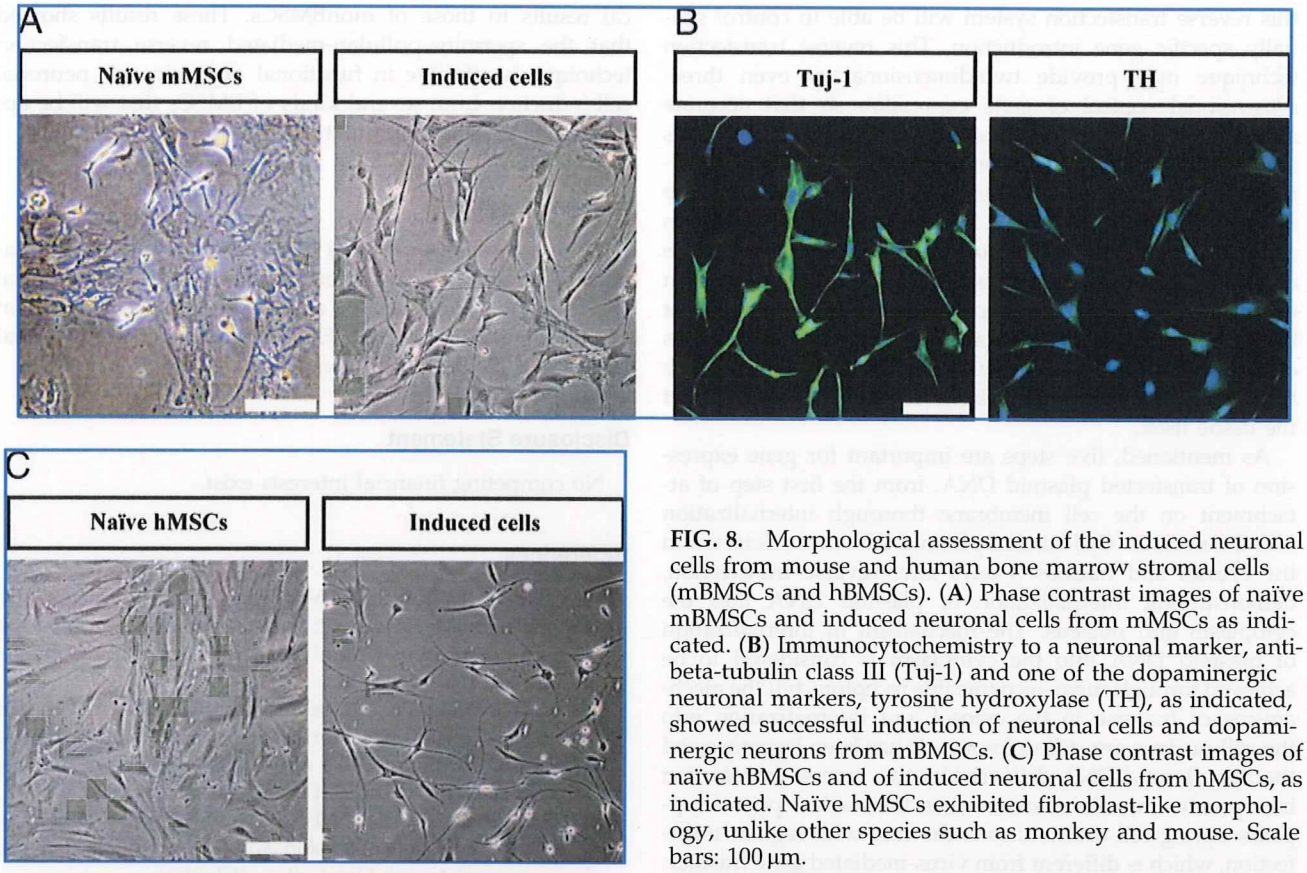

FIG. 8. Morphological assessment of the induced neuronal cells from mouse and human bone marrow stromal cells (mBMSCs and hBMSCs). (**A**) Phase contrast images of naïve mBMSCs and induced neuronal cells from mMSCs as indicated. (**B**) Immunocytochemistry to a neuronal marker, anti-beta-tubulin class III (Tuj-1) and one of the dopaminergic neuronal markers, tyrosine hydroxylase (TH), as indicated, showed successful induction of neuronal cells and dopaminergic neurons from mBMSCs. (**C**) Phase contrast images of naïve hBMSCs and of induced neuronal cells from hMSCs, as indicated. Naïve hMSCs exhibited fibroblast-like morphology, unlike other species such as monkey and mouse. Scale bars: 100 μm.

the induction of dopamine-producing neuronal cells from BMSCs in several kinds of species in which the introduction of NICD plasmid is indispensable for the induction process. This reverse transfection allows transfected cells to survive for further treatment, such as selection by G418. Virtually, sequential treatments comprising reverse transfection, antibiotic selection, and trophic factor stimulation gave the successful induction of mature neuronal cells that have an ability to produce and release dopamine, whose maturation was confirmed using immunocytochemistry and HPLC. Because naïve monBMSCs and TF-monBMSCs did not show neuronal marker expression or dopamine release, it is likely that NICD introduction is required for the effective induction of dopamine-producing cells from BMSCs. These findings also indicated that tangled treatments for induction of specific cells after this spermine-pullulan-mediated reverse transfection can be applied to the multipotent cells to obtain desirable results, which can be attributable to no detrimental effect on differentiation activity. Thus, this reverse transfection method may be suitable to the induction of specific cells for cell therapy from pluri- and multipotent cells.

Control for differentiation and induction of stem cells is an important matter in stem cell biology and regenerative medicine. Genetic manipulations such as gene introduction are key ways to modify the differentiation and function of stem cells.[18,19] In this context, it is necessary to develop a gene delivery system with low cytotoxicity and high efficiency. We showed that this reverse transfection method can be applied to the susceptible cells, to which generally gene transfer is difficult, even with high efficiency. Thus, our data clearly indicate that the spermine-pullulan-mediated reverse transfection is one of the most practical methods of genetically modifying the differentiation of BMSCs, which is also applicable to other kinds of tissue stem cells.

BMSCs offer possibilities for clinical application, because they can be efficiently expanded *in vitro* to achieve a therapeutic scale. They are easily accessible through the aspiration of bone marrow and can easily be expanded on a large scale for auto-transplantation. We can collect BMSCs without encountering serious ethical problems, and there is no need to use a fertilized egg or fetus. Thus, BMSCs are strong and hopeful candidates for use in cell-based therapy. In addition, because BMSCs are obtained from marrow banks, transplantation of induced cells with the same human leukocyte antigen subtype from a healthy donor may minimize the risk of rejection.

Because BMSCs can be obtained from patients, it is possible to establish "auto cell transplantation therapy" using BMSCs. Fortunately, we have confirmed that human serum is more appropriate for the differentiation of human BMSCs than fetal bovine serum (unpublished data). To realize this ideal, it is necessary to develop a practical system of differentiating BMSCs into cells with a purpose. For Parkinson's disease, transplantation of dopaminergic neurons is believed to be effective.[28] Our method would be one possible way to regulate BMSC differentiation into functional dopaminergic neuronal cells, which will be applicable to auto-transplantation therapy in Parkinson's disease.

Taking into account the anchoring of the plasmid DNA on the surface of culture dishes, one possibility is that

this reverse transfection system will be able to control spatially specific gene introduction. This reverse transfection technique may provide two-dimensional or even three-dimensional control of gene expression so that accurate alignment of specific cells can be acquired. In our previous study, we found that high concentration of ProNectin coating on the surface of culture dishes provided more luciferase activity in a dose-dependent manner,[20] indicating that, in addition to changing the promoter of plasmid DNA, the concentration of adhesion substrate can modify the protein expression level. Although application of cell therapy has not been considered adequate for the repair of tissue that is composed of a complex organization of cells, this reverse transfection method might allow the strategy to construct the tissue itself.

As mentioned, five steps are important for gene expression of transfected plasmid DNA, from the first step of attachment on the cell membrane thorough internalization into the nucleus. Cy5-labeled plasmid DNA was detected in the cytosol and nucleus 3 days after reverse transfection, demonstrating internalization of plasmid DNA into the cytoplasm and nucleus. The mechanism of internalization of plasmid DNA into the cytoplasm is considered to be achieved through sugar-recognizable receptors, but the mechanisms of traverse in the cytosol and internalization into the cell nucleus are still unknown. It has been hypothesized that the plasmid DNA delivered by this reverse transfection incorporates into the nucleus when the nuclear pore disappears during cell division, as is the case with regular transfection, which is different from virus-mediated gene transfer. Our induction method for neuronal cells from BMSCs does not need sequential gene manipulation, so this point is not problematic in our system. For application of this reverse transfection method to induce the specific cell under the specific condition, we must be aware of the possibility that functional gene expression transfected using this reverse transfection technique will not be achieved after cells are post-mitotic, even when plasmid DNA is internalized into the cytoplasm.

The plasmid DNA intermingled with spermine-pullulan (PIC) could be easily fixed on the surface of Pronectin- and gelatin-coated culture dishes. For spermine-pullulan-mediated reverse transfection, cells were seeded on the culture dishes fixed with PIC and incubated for 24 h after seeding. Plasmid DNA could penetrate into the cell membrane and the nucleus at 3 days after reverse transfection. Almost all transfected cells survived after transfection, and the expression intensity of exogenous gene was determined using luciferase assay, which showed almost the same level of protein expression as that of Lipofectamine 2000. For this low toxicity, monBMSCs, which are vulnerable to manipulation such as gene introduction and reagent stimulation, were used in our study for dopaminergic neuronal cell induction. Cells were introduced with NICD, selected with G418, and followed using trophic factor treatment. Morphological and immunocytochemical analysis showed the induction of neuronal cells from monBMSCs. Additional GDNF treatment promoted BMSC-derived neuronal cells to dopamine-producing cells; immunocytochemistry showed the expression of dopaminergic neuron marker TH and the secretion of dopamine detected using HPLC. BMSCs derived from mice and humans also demonstrated basically identical results to those of monBMSCs. These results showed that the spermine-pullulan-mediated reverse transfection technique is effective in functional dopaminergic neuronal cell-induction from several kinds of BMSCs that will be applicable to genetic manipulation of stem cells.

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Disclosure Statement

No competing financial interests exist.

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Address reprint requests to:
Mari Dezawa, M.D., Ph.D.
Department of Stem Cell Biology and Histology
Tohoku University Graduate School of Medicine
2-1 Seiryo-machi, Aoba-ku, Sendai 980-8575
Japan

E-mail: mdezawa@m.tains.tohoku.ac.jp

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Committed neural progenitor cells derived from genetically modified bone marrow stromal cells ameliorate deficits in a rat model of stroke

Makoto Hayase[1], Masaaki Kitada[2,3], Shohei Wakao[2,3], Yutaka Itokazu[2], Kazuhiko Nozaki[1,4], Nobuo Hashimoto[1,5], Yasushi Takagi[1] and Mari Dezawa[2,3]

[1]*Department of Neurosurgery, Kyoto University Graduate School of Medicine, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto, Japan;* [2]*Department of Anatomy and Neurobiology, Kyoto University Graduate School of Medicine, Yoshidakonoe-cho, Sakyo-ku, Kyoto, Japan;* [3]*Department of Stem Cell Biology and Histology, Tohoku University Graduate School of Medicine, 2-1, Seiryo-machi, Aoba-ku, Sendai, Japan;* [4]*Department of Neurosurgery, Shiga University of Medical Science, Seta Tsukinowa-cho, Otsu City, Shiga, Japan;* [5]*National Cardiovascular Center, 5-7-1, Fujishiro-dai, Suita City, Osaka, Japan*

Bone marrow stromal cells (MSCs) are an excellent source of cells for treating a variety of central nervous system diseases. In this study, we report the efficient induction of committed neural progenitor cells from rat and human MSCs (NS-MSCs) by introduction of cells with the intracellular domain of Notch-1 followed by growth in the free-floating culture system. NS-MSCs successfully formed spheres, in which cells highly expressed the neural precursor cell markers. The commitment of spheres to neural lineage cells was confirmed by their successful differentiation into neuronal cells when exposed to a differentiation medium. To determine the therapeutic potential of NS-MSCs, cells were transplanted into the cortex and striatum in a rat model of focal cerebral ischemia. The survival, distribution, and integration of NS-MSCs in the host brain were very high, and at day 100, grafted NS-MSCs were positive for dopaminergic, glutamatergic, and γ-amino butyric acid (GABA)ergic neuronal markers. They extended long neurites for nearly 6.3 mm and many of these expressed synaptophysin. Significant behavioral recovery was also observed in limb-placing and water-maze tests. These suggest a high potential for this MSC approach in the replenishment of neural cells for stroke and for a wide range of neurodegenerative conditions that require various types of neural cells.
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Correspondence: Dr Y Takagi, Department of Neurosurgery, Kyoto University Graduate School of Medicine, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto, 606-8507 Japan.
E-mail: ytakagi@kuhp.kyoto-u.ac.jp or
Dr M Dezawa, Department of Stem Cell Biology and Histology, Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan.
E-mail: mdezawa@m.tains.tohoku.ac.jp
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Introduction

Stroke is a major cause of death, followed closely by cancer and myocardial infarction, and is characterized by progressive neurologic deficits. Effective treatments for restoring lost neurologic function are currently lacking (Bliss *et al*, 2007; Lindvall and Kokaia, 2006). Cell transplantation is one potential strategy for the treatment of stroke and other progressive neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease, and others. Embryonic stem cells (ESCs) and neural stem cells (NSCs) have prompted great interest because of their potential to replenish lost neuronal cells (Reubinoff *et al*, 2001). Stem and/or neural progenitor cells derived from the umbilical cord blood, amnion/placenta, and adipose tissues are also candidates for

cell transplantation therapy (Borlongan *et al*, 2004; Igura *et al*, 2004; In 't Anker *et al*, 2003; Lin *et al*, 2006).

Bone marrow contains mesenchymal stem cells, also known as bone marrow stromal cells (MSCs) that possess the potential to differentiate into other cell types (Prockop, 1997; Tang *et al*, 2007). MSCs are promising candidates for clinical application because they are easily isolated from patient bone marrow aspirates and are readily expanded *in vitro* for auto-transplantation without posing major ethical problems (Dezawa *et al*, 2005). Naive MSCs have been reported by several groups to enhance functional recovery after stroke, with the mechanism of action postulated to be mediated by the trophic effects of MSCs rather than by the spontaneous neuronal differentiation of MSCs in the host brain (Chen *et al*, 2001*a*, *b*; Chen and Chopp, 2006; Li *et al*, 2000, 2002; Shen *et al*, 2007).

In the acute stage of brain injury, neuroprotective treatment may effectively prevent the progressive loss of damaged neuronal cells, but in advanced degenerative stages, neuronal replacement is deemed necessary for cell therapy to exert its benefits. We reported earlier that functional dopaminergic neurons can be specifically induced from human MSCs with a very high efficiency without glial development by the Notch intracellular domain (NICD) transfection followed by the administration of a certain combination of trophic factors, including glial cell line-derived neurotrophic factor (CDNF) (PeproTech, Inc., Rocky Hill, NJ, USA) (Dezawa *et al*, 2004; Mimura *et al*, 2005).

In this study, we newly found that rat and human MSCs shift to a neural progenitor-like state, alternatively, committed neural precursor cells, after receiving Notch signal followed by the culture in the free-floating system. Notch intracellular domain-transfected MSCs (NICD-MSCs) successfully formed spheres (NS-MSCs) that highly expressed neural precursor cell markers. The commitment of spheres to neural lineage cells was further evaluated *in vitro*, which showed that sphere-derived cells successfully differentiated into neuronal cells when exposed to a differentiation medium, and expressed neurotransmitters or related markers, as well as released neurotransmitters. However, in contrast to neurospheres grown from the brain, the bone marrow-derived NS-MSCs showed a low capacity for differentiating into astrocytes. When rat MSCs-derived neural progenitor-like cells were transplanted into a rat focal ischemia model, they showed a much higher efficiency survival, distribution, and integration in the host brain compared with using naive MSCs. They became postmitotic and adapted well to the host microenvironment to differentiate into cells that were positive for dopaminergic, glutamatergic, and γ-amino butyric acid(GABA)ergic neuronal cell markers. Significant behavioral recovery was recognized in the limb-placing and water-maze tests. In addition, tumor formation was not observed up to 100 days after transplantation as judged by histologic analysis.

As MSCs are useful cells in that they can be obtained easily from patients or a bone marrow bank and can be expanded in culture with fewer ethical problems, our finding offers a new efficient *in vitro* neural progenitor differentiation method to provide a realistic source of neuronal cells for cell therapy.

Materials and methods

Preparation of Marrow Stromal Cells and Neural Induction

The usage of human MSCs for the experiment was approved by the Kyoto University Graduate School and Faculty of Medicine, Ethics Committee. All animal experiments were approved by the Animal Care and Experimentation Committee of the Kyoto University Graduate School of Medicine. Human MSCs were purchased from Cambrex (East Rutherford, NJ, USA). Rat MSCs were harvested according to methods published earlier (Dezawa *et al*, 2001). Both rat and human MSCs were maintained in α-MEM (α-minimum essential medium) (Sigma, St Louis, MO, USA) containing 10% fetal calf serum (FCS) and kanamycin at 37°C with 5% carbon dioxide (CO_2). Rat and human MSCs were then transfected with a vector (pCI neo-NICD) containing the mouse NICD (the NICD cDNA coded for a transmembrane region that included a small fragment of the extracellular domain, followed by a sequence encoding the entire intracellular domain of mouse Notch, initiating at amino acid 1,703 and terminating at the 3′-untranslated sequence). This fragment was subcloned into a pCI-neo vector (Promega Corp., Madison, WI, USA) and was transfected with MSCs using Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA, USA) and selected by G418 (Invitrogen Corp.) for 7 days, according to the manufacturer's instructions.

Sphere Formation

After G418 selection, both rat and human NICD-transfected cells (referred to as NICD-MSCs) were washed and cultured in α-MEM containing 10% fetal calf serum for 2 days for recovery. After recovery for 2 days, cells were washed and subjected to the free-floating culture for 8 days to generate spheres (referred to as NS-MSCs); i.e., neurobasal medium (Invitrogen Corp.) supplemented with B27 supplement (Invitrogen Corp.), 20 ng/mL bFGF (basic fibroblast growth factor) (R&D Systems Inc., Minneapolis, MN, USA) and 20 ng/mL epidermal growth factor (EGF) (R&D Systems Inc.) at a cell density of 1×10^5 cells per mL on low cell-binding dishes (Nalgene Nunc, Rochester, NY, USA) (Takagi *et al*, 2005). For transplantation, rat spheres, NS-MSCs, were collected and transplanted into the rat (MCAo) middle cerebral artery occlusion model 8 days after the free-floating culture system.

For the differentiation of rat and human NS-MSCs into neuron-like cells *in vitro*, spheres were isolated manually and plated onto laminin (BD Sciences, Bedford, MA, USA) coated slides in a neurobasal medium containing the B27 supplement, 1% fetal bovine serum (FBS) by withdrawal of

EGF and by the addition of 10 μmol/L Forskolin (Calbiochem, La Jolla, CA, USA), 20 ng/mL CNTF (ciliary neurotrophic factor) (R&D Systems Inc.), and 20 ng/mL bFGF (Dezawa *et al*, 2004). After 1 week of culture, cells were fixed and evaluated by immunocytochemistry. To examine the ability to form secondary to fourth spheres, rat and human NS-MSCs were dissociated into single cells and cultured in the free-floating culture system for 8 days in each step.

Induction of Permanent Focal Cerebral Ischemia

Permanent focal cerebral ischemia was induced in 10-week-old male Wister rats using the intraluminal filament technique according to the modified procedure of Koizumi *et al* (1986) and Ohta *et al* (2006). Details of the procedure are available in Supplementary Information.

Transplantation

Three days after MCAo, under inhalational anesthesia with 2% halothane, the rats were fixed in a stereotaxic frame (Narishige, Tokyo, Japan). During the expansion and recovery of rat NICD-MSCs after G418 selection, and green fluorescent protein (GFP) lentivirus was added to the culture medium for 2 days for the labeling of rat NICD-MSCs as described earlier (Nguyen *et al*, 2005; Shimizu *et al*, 2007). After GFP-lentivirus infection, they were subjected to the free-floating culture system to generate rat NS-MSCs, and were injected as spheres into the following places using an electric injector (Muromachi Kikai Co., Tokyo, Japan): 3.5×10^4 cells per 7 μL of rat NS-MSCs into the striatum (from the bregma: anterior (A) 0.0 mm, right (R) +2.0 mm, ventral (V) −4.5 mm, incisor bar −3.3 mm) and 1.5×10^4 cells per 3 μL of those (from the bregma: A 0.0 mm, R +2.0 mm, V −2.0 mm, incisor bar −3.3 mm) into the cortex along the same tract using a Hamilton microsyringe (Hamilton Company, Reno, NV, USA) fitted with a 26-gauge blunt needle. For the control experiment, naive rat MSCs transplantation (cells which were dissociated into a single cell with trypsin were transplanted in the same manner as described above) and vehicle injection (7 and 3 μL of α-MEM into the striatum and cortex, respectively) were performed. The distribution of animals was NS-MSCs ($n = 13$), MSCs ($n = 9$), vehicle ($n = 11$).

Measurement of Lesion Volume

At 100 days after transplantation, animals were perfused through the heart with cold saline and 4% paraformaldehyde in 0.1 mol/L PBS (phosphate-buffered saline) under deep anesthesia (overdose of pentobarbiturate). The brains were cut coronally at 2-mm intervals. Detailed methods of calculating the lesion volume are described in Supplementary Information.

Retrograde Tracer Labeling

Retrograde tracer labeling with Fluorogold (Molecular Probes, Carlsbad, CA, USA) was performed as described earlier (Hayashi *et al*, 2006). Briefly, at 7 days before transcardiac perfusion, the rats received an injection of 0.1 μL of hydroxyl stilbamidine (equivalent to Fluorogold) into the substantia nigra (from the bregma: A −5.0 mm, R +2.3 mm, V −8.25 mm incisor bar −3.3 mm) on the grafted side through a 26-gauge Hamilton microsyringe under general anesthesia.

Antibodies

Primary antibodies used in immunocytochemistry and immunohistochemistry are listed in Table 1. Secondary

Table 1 List of primary antibodies

Primary antibody	*Secondary antibody*	*Company*	*Dilution*
Green fluorescent protein (GFP)	Rabbit IgG	Abcam, Cambridge, MA, USA	1:400
Green fluorescent protein (GFP)	Mouse IgG	Molecular Probes	1:500
β-Tubulin class III (Tuj-1)	Mouse IgG	Sigma	1:800
MAP2ab	Mouse IgG	Sigma	1:1,000
Parvalbumin	Mouse IgG	Sigma	1:2,000
γ-Amino butyric acid (GABA)	Rabbit IgG	Sigma	1:200
Glial fibrillary acidic protein (GFAP)	Rabbit IgG	DAKO, Caspenteria, CA, USA	1:300
Glial fibrillary acidic protein (GFAP)	Mouse IgG	Chemicon	1:200
Sox2	Rabbit IgG	Chemicon	1:200
NeuroD	Rabbit IgG	Chemicon	1:200
NeuN	Mouse IgG	Chemicon	1:200
Tyrosine hydroxylase (TH)	Rabbit IgG	Chemicon	1:1,000
Dopamine transporter (DAT)	Rabbit IgG	Chemicon	1:200
Glutamate	Rabbit IgG	Chemicon	1:200
Calbindin	Rabbit IgG	Chemicon	1:500
DARPP32	Rabbit IgG	Chemicon	1:500
Choline acetyl transferase (ChAT)	Rabbit IgG	Chemicon	1:1,000
Serotonin (5-HT)	Rabbit IgG	ImmunoStar	1:2,000
Glutamic acid decarboxylase (GAD)	Rabbit IgG	Chemicon	1:1,000
Synaptophysin	Mouse IgG	Chemicon	1:1,000
Nestin	Mouse IgG	BD Pharmingen, San Jose, CA, USA	1:400
Ki-67	Rabbit IgG	Thermo, Waltham, MA, USA	1:200

IgG, immunoglobulin G; MAP2ab, microtubule associated protein 2.

antibodies used were antirabbit IgG (immunoglobulin G) conjugated either to Alexa Fluor 488 or 568, and antimouse IgG conjugated either to Alexa Fluor 488 or 568 (1:500; Molecular Probes, Carlsbad, CA, USA).

Immunocytochemistry

Differentiated cells from rat and human NS-MSCs were fixed with 4% paraformaldehyde in 0.1 mol/L PBS. Spheres derived from rat and human MSCs were fixed with 4% paraformaldehyde, cryoprotected in a series of sucrose solutions (15, 20, and 25% sucrose in 0.02 mol/L PBS) at 4°C for 2 days, and then cut into 10-μm-thick sections using cryostat (Leica CM 1850, Wetzlar, Germany). A detailed procedure of immunocytochemistry is described in Supplementary Information.

The total number of cells was evaluated by counting of 4′, 6-diamidino-2-phenylindole- (DAPI)-positive nuclei, and the percentage of immunoreactive cells was evaluated using the Nikon confocal microscope system C1si (Nikon Corporation, Tokyo, Japan). The cells in five fields, which include 100 to 500 cells, were counted for three independent cultures.

Western Blot

pCI-neo-NICD-GFP vector was constructed by the insertion of EGFP gene fragment (Promega Corp.) into the pCI-neo-NICD vector. Six, 12, 24, 36, 48 h and 3 and 5 days after the introduction of pCI-neo-NICD-GFP plasmid by Lipofectamine 2000, cells were harvested and subjected to western blot analysis as described in Supplementary Information. G418 was administrated from 48 h to 5 days after lipofection for the selection of the transfected cells.

Reverse Transcription-PCR

To analyze the relative expression of different mRNAs, the amount of cDNA was normalized on the basis of the signal from ubiquitously expressed β-actin. Primer sequences and precise conditions are β-actin: 5′-AACTGGGACGATATG GAGAA-3′ (forward) and 5′-GTAACCCTCATAGATGGG CA-3′(reverse) (TM, melting temperature; 66°C, 25 cycles); neuron-specific enolase (NSE): 5′-GGTGAAGGAAGCCA TTGACAA-3′ (forward) and 5′-ATGCCGACATTGGCTGT GA-3′ (reverse) (TM, melting temperature; 66°C, 27 cycles); and choline acetyltransferase (ChAT): 5′-GGAGCTAT TCCTCTTTCGGGATT-3′ (forward) and 5′-GTCAGTCATG GCTTGCACAAA-3′ (reverse) (TM; 66°C, 40 cycles). Total RNA of rat adult brain was used in the reaction of the positive control for each factor.

HPLC (High Performance Liquid Chromatograph)

NS-MSCs cultured in the free-floating culture system for 7 days were replated and cultured in α-MEM with 1% serum, 10 μmol/L Forskolin, 20 ng/mL CNTF, and 20 ng/mL bFGF, followed by the administration of glial cell line-derived neurotrophic factor (50 ng/mL) for 5 days. The concentration of dopamine release was determined by HPLC using a reverse-phase column and an electrochemical detector system (Eicom, Kyoto, Japan) as described earlier (Dezawa *et al*, 2004). Details of the procedure are available in Supplementary Information.

Behavioral Analysis

The limb-placing test was a modified version of a test described by De Ryck *et al* (1989) and Jeong *et al* (2003). The test was performed the day before ischemia induction and after transplantation on days 4, 10, 14, 21, 28, 56 and 84. The total score ranged from 0 to 7.

The Morris water-maze test was performed as described earlier (Mimura *et al*, 2005; Morris, 1984). This test was performed from day 96 to day 100 after transplantation. Each detailed procedure is described in Supplementary Information. After behavioral follow-up, at 100 days after transplantation, each animal underwent perfusion fixation under anesthesia. In all, 10-μm-thick brain cryosections were prepared by cryostat. As for the positive control, normal rats ($n = 8$) were used.

Immunohistochemistry

In 14 and 100 days after transplantation, each animal was perfused through the heart with cold saline and 4% paraformaldehyde in 0.1 mol/L PBS under deep anesthesia (overdose of pentobarbiturate). The brains were cut coronally at 2-mm intervals, and 6 brain slices were prepared totally. Each brain slice was cut into 10-μm-thick coronal sections using a cryostat, and one cryosection was chosen from each slice. Transplanted cells of 10 fields in each cryosection were counted using the Nikon confocal microscope system C1si for three animals, and the cell number of positive cells in serial sections was calculated. Cells of thickness larger than 10-μm could have been counted multiple times. For this, the size and thickness of cells were corrected using the Abercrombie method for controlling the bias in counting (Abercrombie, 1946).

Details of the immunostaining procedure are described in Supplementary Information. The samples were inspected under Nikon confocal microscope system C1si.

The numbers of animals subjected to experiments were NS-MSCs = 13, MSCs = 9, vehicle = 11 (for 100 days after transplantation), and NS-MSCs = 8, MSCs = 8 (for 14 days after transplantation).

Real-Time PCR

Total RNA was collected using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany), and cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Primers and probes for NSE (γ, γ-enolase), sodium channel voltage-gated type III Scn3a, and β-actin were purchased from TaqMan Gene Expression Assay (Applied Biosystems) and subjected to real-time PCR according to the manufacturer's instructions.

To estimate the number of transplanted cells that survived and integrated in the host tissue 100 days after

grafting, real-time PCR was performed using a 7300 sequence detection system (Applied Biosystems). Real-time PCR primers and the probe for GFP used in this study were 5′-AGTCCGCCCTGAGCAAAGA-3′ and 5′-TCCAGC AGGACCATGTGATC-3′, and 5′-FAM-CCCAACGAGAAG CG-MGB-3′, respectively. Standard curves were generated by serially diluting cDNA derived from cultured MSCs. Values were normalized by assays for β-actin using TaqMan β-actin Detection Reagents (Applied Biosystems).

Statistical Analysis

Data are expressed as mean ± s.e.m. Data were compared using ANOVA (analysis of variance) with pairwise comparisons by the Bonferroni method. As the score of the limb-placing test is ordinal (i.e., 8 levels (0 to 7)), the Mann–Whitney *U*-test was chosen for the nonparametric statistical analysis of the limb-placing test.

Results

Generation of Spheres from Marrow Stromal Cells

After NICD transfection into MSCs (NICD-MSCs), morphologic changes were observed, i.e., the cells became smaller (Figures 1A and 1B). These NICD-MSCs were subjected to the free-floating culture system (neurobasal medium supplemented with B27, bFGF, and EGF for 8 days) to form spheres (NS-MSCs, Figure 1C). Naive MSCs formed spheres but the efficiency was very low compared with that of both rat and human NICD-MSCs; in rats, NICD-MSCs produced seven times more spheres (~35 spheres per 10,000 cells) than did naive MSCs (~5 spheres per 10,000 cells), and in humans, NICD-MSCs produced ~50 spheres per 10,000 cells in all clones examined, whereas naive MSCs produced 0 to 4 spheres per 10,000 cells. The diameter of the NS-MSCs spheres was ~60 to 150 μm, whereas that of MSCs was mostly under 60 μm. Rat and human NS-MSCs were dissociated and subjected to secondary, third, and fourth sphere formation. Eight days after replating, secondary spheres could be recognized but few third and no fourth generation were observed (data not shown). In this regard, the sphere formation of NS-MSCs is limited.

To determine whether the spheres contained cells with neural progenitor properties, we examined the expression of Sox2, nestin, and NeuroD. In naive rat MSCs, a few cells were positive for Sox2 (2.0 ± 1.4%), nestin (2.3 ± 0.9%), and NeuroD (9.4 ± 2.2%). In contrast, induced spheres contained much higher percentages of cells positive for nestin (61 ± 4.8%), NeuroD (96.8 ± 0.9%), and Sox2 (93.9 ± 2.4%) (Figures 1D–1F). Almost identical data were obtained from human MSCs.

To examine whether cells in the spheres were committed to a neural lineage cell, spheres were dissociated, replated to adherent dishes, and then differentiated *in vitro* by low serum and withdrawal

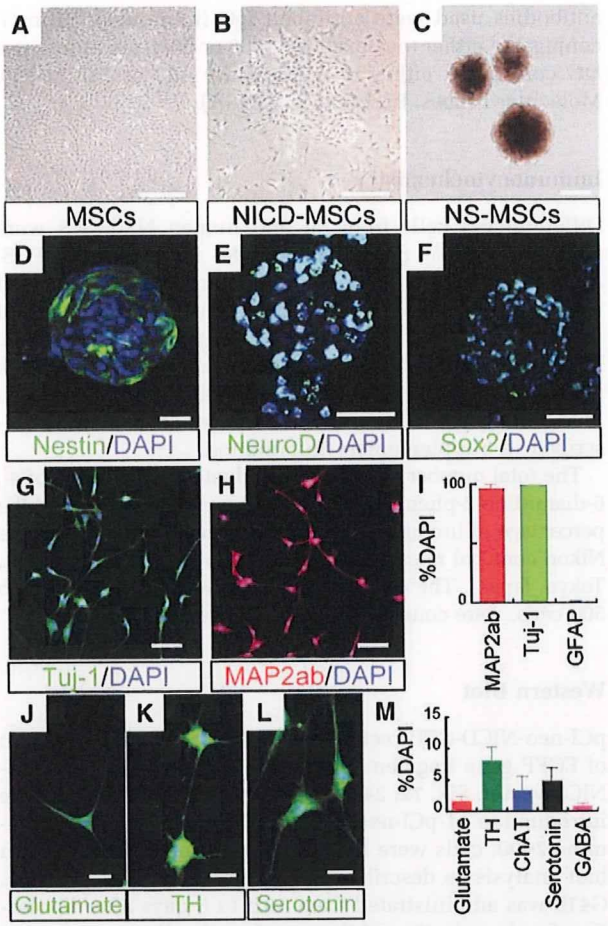

Figure 1 Neuronal induction from marrow stromal cells (MSCs). (**A–C**) Phase-contrast images of rat cells. After Notch intracellular domain (NICD) transfection, original naive MSCs (panel A) changed their morphology (NICD-MSCs, panel B). Panel C represents spheres made from NICD-MSCs (refereed to as NS-MSCs) on a low cell-binding dish. (**D–F**) Expression of nestin (panel D), NeuroD (panel E), and Sox2 (panel F) in rat NS-MSCs. (**G** and **H**) β-tubulin isotype III (Tuj-1)- (panel G) and microtubule associated protein 2 (MAP2ab)-positive (panel H) neuron-like cells differentiated from rat NS-MSCs. (**I**) The proportions of rat cells expressing neural markers. (**J–L**) Expression of glutamate (panel J), tyrosine hydroxylase (TH) (panel K), and serotonin (panel L) in neuron-like cells derived from rat NS-MSCs spheres. The proportions of cells expressing neurotransmitter-related markers in rat cells (**M**). Scale bars = 250 μm (panels A and B), 100 μm (panel C), 50 μm (panels D–F), 20 μm (panels J–L).

of EGF. Adhered rat and human cells had neurite-like processes with abundant varicosities and were immunoreactive to markers of neuronal cells, β-tubulin isotype III (Tuj-1) (98.5 ± 0.5%) and microtubule associated protein 2 (MAP-2) (95.7 ± 3.7%) (Figures 1G–1I). A very small number of cells were immunopositive for the astrocyte marker, glial fibrillary acidic protein (0.7 ± 0.3%). The expression of transmitter-related markers in rat cells was 1.6 ± 0.7% (glutamate), 7.9 ± 2.2% (TH (tyrosine hydroxylase)), 3.2 ± 2.3% (ChAT), 4.7 ± 2.3%

(serotonin), and 0.7 ± 0.4% (GABA) (Figures 1J–1M), and that in human cells was 8.2 ± 5.7% (glutamate), 8.9 ± 4.1% (TH), 4.7 ± 2.4% (ChAT), 3.8 ± 2.9% (serotonin), and 3.8 ± 3.7% (GABA), suggesting their mature neuronal subtypes. We also confirmed their expression of neuronal markers in RT-PCR and real time-PCR. The clear upregulation of NSE after neuronal induction from NS-MSCs was recognized in RT-PCR (Supplementary Figure 1). They expressed not only NSE but also ChAT, one of the markers for functional neurons. In real-time PCR, both NSE and voltage-gated sodium channel type III Scn3a were shown to be substantially upregulated or expressed in neuronal cells induced from NS-MSCs but not in naive MSCs (Supplementary Figure 2). In the HPLC measurement, neuronal cells induced from human and rat NS-MSCs showed 1.0 and 0.5 pmol/L per 10^6 cells of dopamine release into the culture media in response to high potassium depolarizing stimuli, respectively, whereas dopamine release from naive human and rat MSCs was under the detection level.

As the introduction of an exogenous gene is an extra hurdle for the safety aspect in the cell transplantation therapy, we tracked the expression of the exogenous *NICD* gene after transfection with pCI-neo-NICD-GFP genes at protein level by western blot in both rat and human MSCs. The NICD-GFP expression was detectable from 12 h after introduction and reached to the maximum expression from 24 to 48 h, and returned to the basal level by 5 days (data not shown), suggesting that the introduced exogenous NICD does not remain within rat and human MSCs for a longer period.

Transplantation of NS-MSCs into Focal Cerebral Ischemic Lesions

To evaluate whether NS-MSCs were able to integrate into the host brain and contribute to the amelioration of the neurodegeneration, we transplanted the rat NS-MSCs into a rat model of permanent focal cerebral ischemia. All rats exposed to focal cerebral ischemia had areas of severe corticostriatal infarction. Each animal received 50,000 cells, which were transplanted into the striatum and cortex after 3 days of MCAo. For control groups, the same number of naive MSCs (MSCs) or vehicle was injected.

The tissue defect observed in each experimental group was defined as the lesion. The mean lesion volumes in the NS-MSCs, MSCs, and vehicle groups on day 100 were 27.1 ± 4.3%, 23.0 ± 2.3%, and 31.4 ± 5.0%, respectively. There was no statistical difference among groups.

Limb-Placing Test

Rats with focal cerebral ischemia initially showed severe impairment in the limb-placing test (Figure 2A). From days 4 to 84 after transplantation,

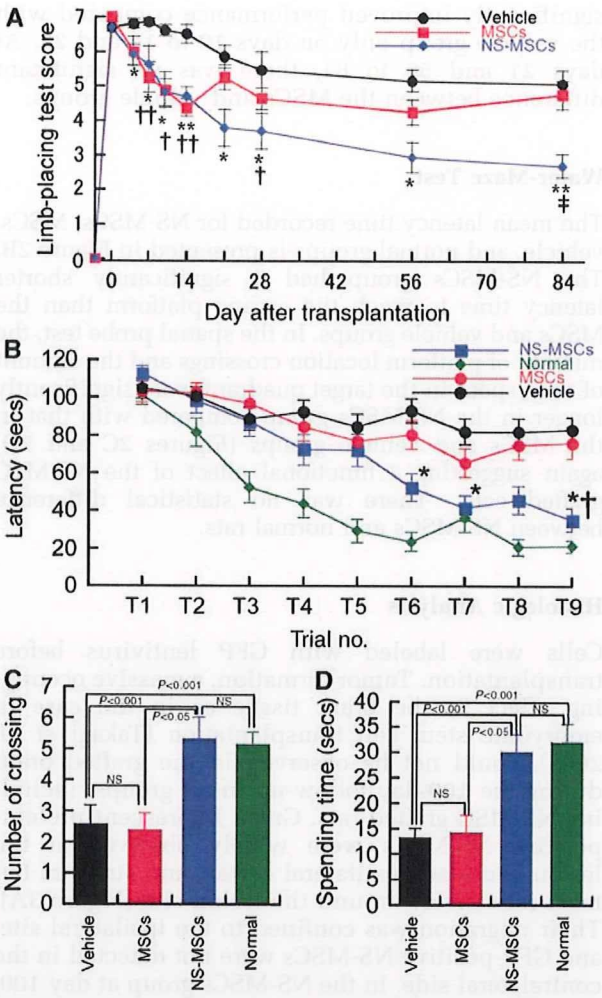

Figure 2 Behavioral analysis. (**A**) Limb placing. Mean scores for the NS-MSCs group were statistically different from those of the marrow stromal cells (MSCs) group at day 84. Mean scores for the MSCs group were also significantly different from those of the vehicle group from days 10 to 14 and at day 28, but not at day 21 and from days 56 to 84. (*NS-MSCs versus vehicle, †MSCs versus vehicle, ‡NS-MSCs versus MSCs, $P < 0.05$, **,††, $P < 0.001$) (**B**) Morris water-maze test. The mean latency of the NS-MSCs group to locate the platform was significantly different from that of the MSCs (*) or vehicle (†) groups. (*,†$P < 0.05$, **$P < 0.001$) (**C**) The result of water-maze spatial probe trial. The duration of time the rat swam through the area, in which the platform was located earlier. (**D**) The time spent in the quadrant, in which the platform was located earlier in the water maze. These values for the NS-MSCs group are significantly different from those of the MSCs or vehicle groups. NS, nonsignificant.

the mean score of the NS-MSCs group was significantly improved compared with that of the vehicle group, indicating that the NS-MSCs group significantly induced long-term behavioral recovery. The mean score of the NS-MSCs group was also significantly improved compared with that of the MSCs group at day 84. In contrast, the MSCs group showed

significantly improved performance compared with the vehicle group only on days 10 to 14 and 28. At days 21 and 56 to 84, there was no significant difference between the MSCs and vehicle groups.

Water-Maze Test

The mean latency time recorded for NS-MSCs, MSCs, vehicle, and normal groups is presented in Figure 2B. The NS-MSCs group had a significantly shorter latency time to reach the escape platform than the MSCs and vehicle groups. In the spatial probe test, the number of platform location crossings and the amount of time spent in the target quadrant were significantly longer in the NS-MSCs group compared with that in the MSCs and vehicle groups (Figures 2C and 2D) again suggesting a functional effect of the NS-MSC grafted cells. There was no statistical difference between NS-MSCs and normal rats.

Histologic Analysis

Cells were labeled with GFP lentivirus before transplantation. Tumor formation, a massive occupying effect in the brain tissue as in the case of embryonic stem cell transplantation (Takagi *et al*, 2005), could not be observed in the grafted brain during the 100-day follow-up in all groups, including NS-MSC grafted rats. Green fluorescent protein-positive NS-MSCs were widely observed at the lesion boundary, ipsilateral cortex, and striatum but most intensively around the lesion site (Figure 3A). Their migration was confined to the ipsilateral site, and GFP-positive NS-MSCs were not detected in the contralateral side. In the NS-MSCs group at day 100, $21 \pm 1.3 \times 10^4$ GFP-labeled cells survived. On the basis of the initial number of transplanted cells (5×10^4), the NS-MSCs appeared to have proliferated after transplantation. Among the GFP-labeled cells, $4.7 \pm 0.02\%$ were immunoreactive for Ki67 at day 14, but Ki67-positive cells were not observed at 100 days, suggesting that the cells did not continue to divide. In the MSCs group, in contrast, there were $1.3 \pm 0.46 \times 10^4$ GFP-positive cells at day 100 and these were mostly detected around the injected area, and rarely observed to be migrating through the parenchyma in the cortex or the striatum (Figure 3A). Furthermore, a successful neuronal differentiation was not observed in the MSCs group. To further confirm the difference in GFP-positive cell numbers between the NS-MSCs and MSCs groups, we performed real-time PCR for the GFP gene. At day 100, the NS-MSCs-transplanted brain showed a 19.6 ± 6.9 times greater GFP gene content compared with the MSCs-transplanted brain.

To determine whether transplanted cells expressed neural markers, sections were immunostained with NeuN and glial fibrillary acidic protein antibodies. In the NS-MSCs group, the percentages of GFP-positive cells that were NeuN- and glial fibrillary acidic protein-positive were $79.5 \pm 0.1\%$ (Figures 3B–3D) and $1.9 \pm 0.03\%$ (data not shown), respectively. In the MSCs group, neither NeuN- nor glial fibrillary acidic protein-positive cells were detected (data not shown). The number of GFP-positive cells in the NS-MSCs and MSCs groups in each section was counted from the anterior to the posterior of the brain. Remarkably, NS-MSCs were observed widely in the antero-posterior axis throughout the brain, in addition to the transplanted site, whereas only a small number of transplanted MSCs were identified, and these were located only around the lesion.

The expression of differentiation markers and neurotransmitter-related markers in NS-MSCs was then examined. The GFP-positive cells were also immunoreactive to dopamine transporter ($32.8 \pm 11.4\%$), TH ($41.4 \pm 17.6\%$), glutamic acid decarboxylase ($10.2 \pm 5.7\%$), glutamate ($4.1 \pm 0.4\%$), calbindin ($7.7 \pm 2.5\%$), DARPP32 ($13.1 \pm 12.5\%$), and parvalbumin ($18.9 \pm 9.9\%$) (Figure 4).

Fluorogold Tracing and Synaptophysin Staining

To assess the possibility that the transplanted NS-MSCs extended neurites with active transport capacity in the host brain, we injected a retrograde tracer at 93 days after transplantation. Fluorogold was injected into the substantia nigra, and then the grafted area was subjected to Fluorogold detection at 100 days. Of the GFP-positive cells, $35.2 \pm 7.3\%$ of the cells were Fluorogold-positive and had a neuron-like morphology (Figures 5A–5F). We also investigated the expression of synaptophysin, and $15.78 \pm 2.94\%$ of GFP-positive cells showed synaptophysin positivity in the striatum (Figures 5G–5J). These data suggest that grafted cells extended long processes from the striatum to the substantia nigra and some of these expressed the synaptic marker synaptophysin.

Discussion

In this study, we report that committed neural progenitor cells, NS-MSCs, can be efficiently differentiated from rat and human MSCs by NICD introduction followed by the free-floating culture system that promotes the formation of spheres. Cells in these spheres expressed markers related to neural progenitor cells, and were shown to be committed to neural lineage cells by immunocytochemistry and grafting into the brain. NS-MSCs also differentiated into neuronal cells after transplantation into a rat focal cerebral ischemia model, and were shown to be immunoreactive to various neurotransmitter-related markers within the host tissue, suggesting their potential to differentiate into various types of neuronal cells. Their contribution to functional recovery was also observed for up to 100 days after transplantation. Importantly, no tumors were detected. On the basis of these results, NS-MSCs may

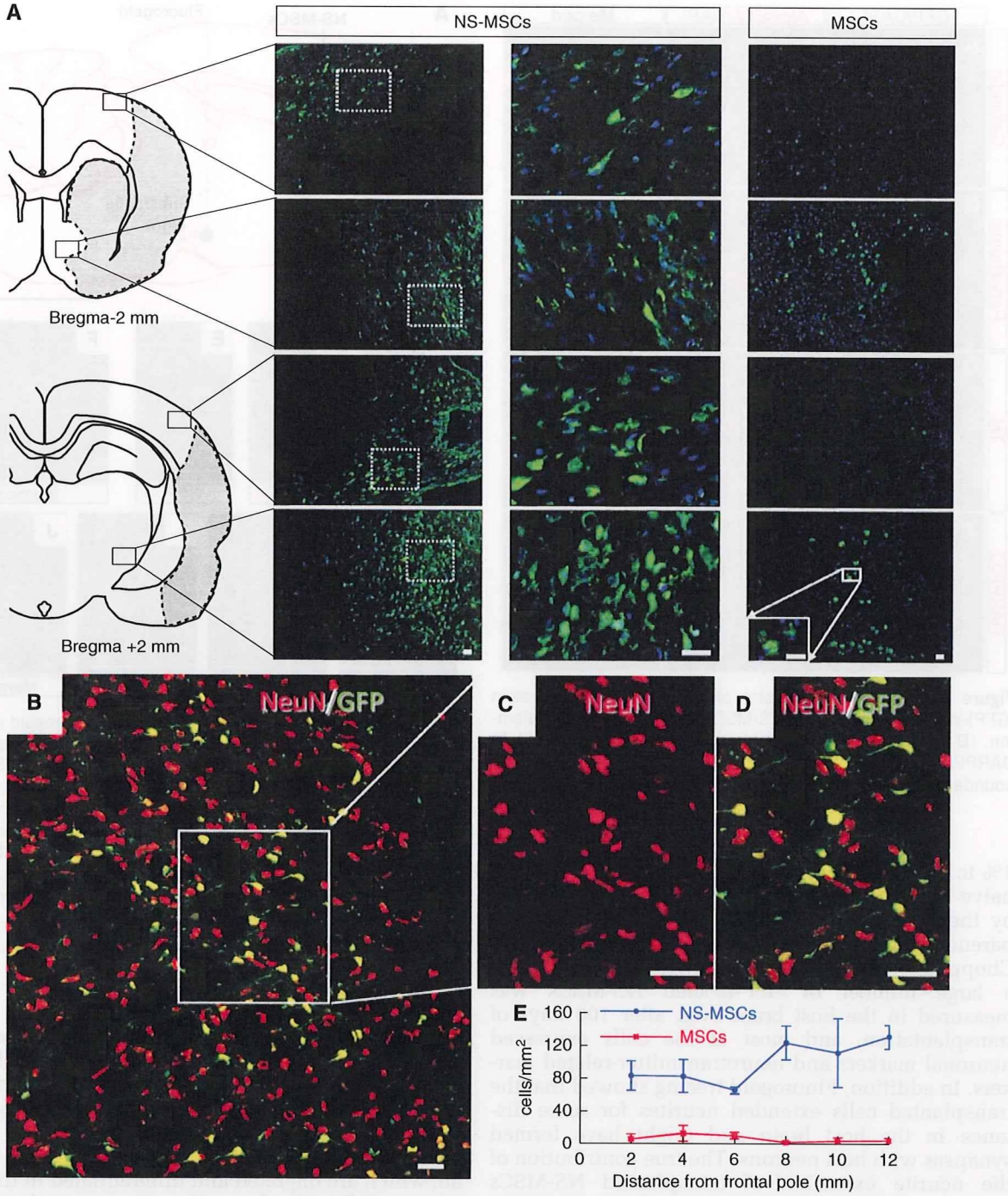

Figure 3 Transplanted cells in the brain lesion (day 100). (**A**) Distribution of transplanted NS-MSCs and marrow stromal cells (MSCs). In the ischemic boundary zone, a number of green fluorescent protein (GFP)-positive cells were observed in the NS-MSCs group. In the MSCs group, a small number of GFP-positive cells were observed, mostly small cells without extended processes. (**B**–**D**) NeuN-positive cells in NS-MSCs. (**E**) The number of GFP-positive cells in NS-MSCs and MSCs groups in each section from the anterior pole to the dorsal side of the brains. Scale bars = 20 μm.

be a good source of neuronal lineage cells for cell transplantation therapy in stroke.

Accumulating evidence shows that naive MSCs transplantation into the ischemic brain leads to improved behavior (Chen and Chopp, 2006; Li *et al*, 2002). According to these reports, the survival rate of MSCs in the host brain was under 10%, and the proportion showing neuronal differentiation was