(Hofstetter et al., 2002; Wu et al., 2003; Ohta et al., 2004), stroke (Chopp and Li, 2002), experimental autoimmune encephalomyelitis (EAE) (Zappia et al., 2005; Zhang et al., 2005, 2006; Gerdoni et al., 2007), and amyotrophic lateral sclerosis (Mazzini et al., 2006). This effect is considered to be produced mainly by soluble factors secreted by MSCs, rather than differentiation of MSCs into the neural cells. Recent studies have elucidated the mechanisms of tissue repair by MSCs: MSCs have an effect on inhibition of apoptosis in several cell types, such as neurons (Crigler et al., 2006; Scuteri et al., 2006) and tumor cells (Ramasamy et al., 2007). MSCs are reported to express neurotrophins, such as nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), which play at least a partial role in promoting cell survival of neurons (Crigler et al., 2006). Another mechanism of MSCs for tissue repair is recruiting endogenous progenitor cells. Munoz et al. showed the evidence that bone marrow cells promote neurogenesis from the endogenous population of neural stem cells (Munoz et al., 2005). In addition, there has been a report in which authors demonstrated the possibility of grafted MSCs to enhance the reconstruction of the neuronal network with functional synaptic transmission by endogenous Purkinje cells in an animal model of neurodegeneration (Bae et al., 2007).

Recently, some interesting functions of MSCs have been reported: cell fusion of infused MSCs to the endogenous cells (Terada et al., 2002; Ying et al., 2002), and mitochondrial transfer to the cells with unfunctional mitochondria (Spees et al., 2006). Spees et al. used human MSCs and skin fibroblasts with the epithelial cell line with nonfunctional mitochondria (A549r0 cells) to show the rescue of A549r0 cells with aerobic respiration. All the rescued clones of A549r0 cells contained functional mitochondrial DNA derived from MSCs without any contamination of genomic DNA from MSCs, indicating that this rescue was not given by cell fusion (Spees et al., 2006). Precise manipulation of these properties of MSCs will give the further outcome from MSC transplantation.

Differentiation ability of MSCs

MSCs have been reported to differentiate into mesenchymal lineage cells, such as osteocytes, chondrocytes, and adipocytes, and some of these differentiation systems are already applied for clinical therapy, such as bone regeneration (Prockop, 1997; Kawate et al., 2006) (Fig. 1). Recently, however, unorthodox plasticity of MSCs has been described in that they have an ability to cross oligolineage boundaries, which were previously thought to be uncrossable. Makino et al. showed that rhythmically contracting cardiomyocytes with expressing cardiac
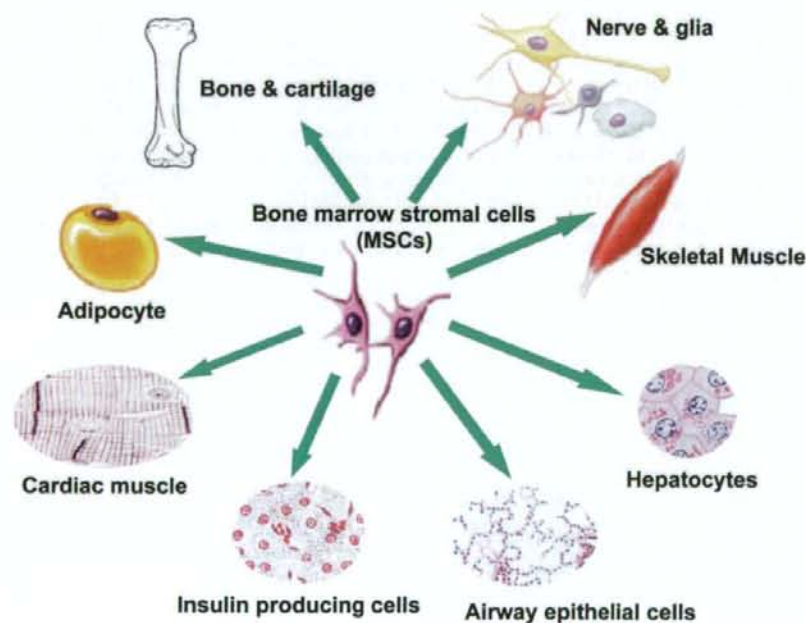

Fig. 1. Differentiation ability of MSCs into different kinds of cells.

muscle markers and electrophysiological characters could be induced from MSCs in vitro (Makino et al., 1999). Hepatocytes, insulin-producing cells and airway epithelial cells, are reported to be inducible from MSCs (Wang et al., 2004, 2005; Choi et al., 2005) (Fig. 1). Recent studies have demonstrated that even kidney tissues can be artificially given rise to from human MSCs (Yokoo et al., 2005, 2006). Accordingly, the potential of MSCs to differentiate from mesenchymal lineages to other lineages is now of interest. As to neurons, previous reports described that MSCs can differentiate into neuron-like morphology only by the administration of reducing agents and/or trophic factors (Sanchez-Ramos et al., 2000; Woodbury et al., 2000; Jiang et al., 2002). However, some other reports expressed skepticism about these observations that simple treatment of MSCs only by reducing agents or factors do not fully induce their differentiation into functional neurons, and that these cells do not actually integrate into the host tissue to contribute to the functional recovery (Neuhuber et al., 2004; Tondreau et al., 2004; Lu and Tuszynski, 2005).

Unlike ES and tissue stem cells, MSCs can be collected without touching serious ethical problems, and there is no need to use fertilized egg or fetus. Thus, MSC are the strong and hopeful candidate for use in cell-based therapy. MSCs thus offer great potential for cell transplantation therapy, while their practical application to human diseases is dependent on the ability to control their differentiation into certain functional cells with high efficiency and purity.

Recently, we have found a method to systematically induce peripheral glial cells, neurons and skeletal muscle lineage cells from human and rat MSCs on a therapeutic scale (Dezawa et al., 2001, 2004, 2005) (Fig. 2). The following sections focus on the differentiation of MSCs into neural and muscle cells, and discuss the possibility of clinical application in neurodegenerative and muscle degenerative diseases.

Induction of cells with Schwann cell property from MSCs

Peripheral glial cells, Schwann cells, which constitute the peripheral nervous system (PNS), are myelin forming cells and are known to support axonal regeneration after damage by providing various kinds of trophic factors and molecular footholds to reconstruct myelin that contribute to functional recovery (Dezawa and Adachi-Usami, 2000; Duboy, 2004; Edgar and Garbern, 2004). Not only in PNS, Schwann cells also support axonal regeneration and reconstruction of myelin in the central nervous system (CNS). For these reasons, they are "cells with a purpose", and represent one of the good candidates for implantation to support regeneration both of PNS and CNS, particularly in spinal
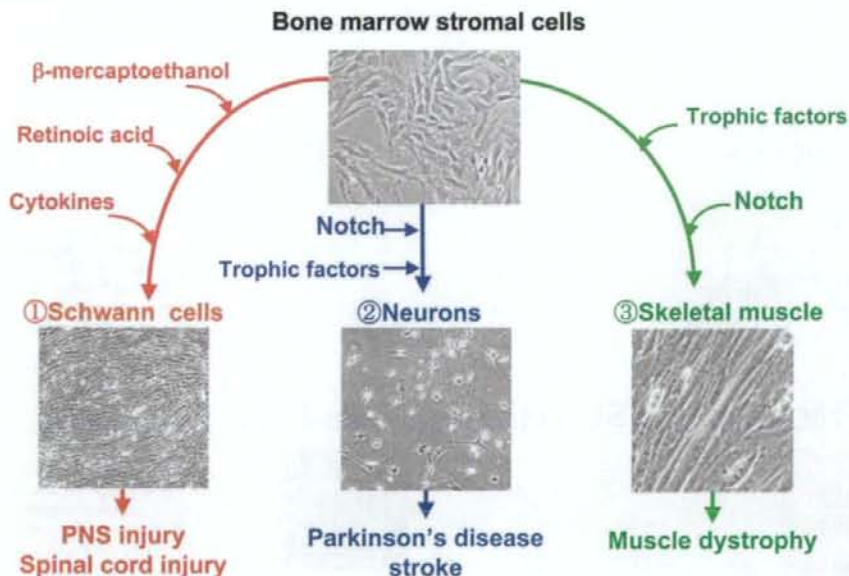

Bone marrow stromal cells

β-mercaptoethanol
Retinoic acid
Cytokines

Trophic factors
Notch

Notch →
Trophic factors →

①Schwann cells   ②Neurons   ③Skeletal muscle

PNS injury
Spinal cord injury   Parkinson's disease
stroke   Muscle dystrophy

Fig. 2. Induction system of Schwann cells, neurons, and skeletal muscle cells in MSCs by combining trophic factor or cytokine treatment with Notch intracellular domain introduction. Induced cells are applicable to PNS and spinal cord injury models, Parkinson's disease and stroke models, and muscle dystrophy model.

cord injury.

Although Schwann cells are hopeful cells, there is a difficulty for clinical use to obtain a sufficient amount of cells. Besides, Schwann cells cannot be harvested unless some extent of healthy peripheral nerve is damaged. Thus, it would be more desirable to establish cells of Schwann cell characteristic from sources which are easily accessed and capable of rapid expansion. We focused on MSCs, and finally established a method to induce MSCs with Schwann cell properties (Dezawa et al., 2001) (Fig. 2).

MSCs were treated with beta-mercaptoethanol (BME) followed by the retinoic acid (RA) treatment and finally administrated cytokines related to Schwann cell differentiation, namely forskolin (known to up-regulate intracellular cAMP; FSK), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF) and neuregulin (Dezawa et al., 2001). Treated cells were morphologically similar to Schwann cell morphology, and expressed p75, GFAP, S-100, O4, P0 and Krox20, all known as markers of Schwann cells (Dezawa et al., 2001). Approximately 97% of the induced cells were positive for Schwann cell markers.

Recovery in nerve and spinal cord injury model by induced Schwann cells

These MSC-derived Schwann cells (M-Schwanns) are effective in promoting axonal regeneration and functional recovery in completely transected adult rat sciatic nerve and spinal cord (Mimura et al., 2004; Kamada et al., 2005; Shimizu et al., 2007). Importantly, human M-Schwanns expressed above mentioned Schwann cell markers and contributed to axonal regeneration, re-myelination and functional recovery when transplanted into rat sciatic nerve injury under the control of immunosuppressant (Shimizu et al., 2007)

M-Schwanns were also effective in the spinal cord injury model. When rat M-Schwanns were transplanted to the defective site of the completely transected model

(the T7 spinal cord segment was completely removed), cells had integrated well into the host spinal cords. In contrast to the control group that had received only matrigel in the deficient site, the number of neurofilament-positive nerve fibers was significantly larger in the M-Schwann group. The large majority of these nerve fibers were revealed to be tyrosine hydroxylase (TH)-positive fibers, while some of CGRP- and serotonin-positive fibers were also contained (Kamada et al., 2005). Hindlimb function recovered in the M-Schwann group from 4 weeks after transplantation, and a significant difference was recognized in BBB score up to 6 weeks after transplantation. The best recovery score in the M-Schwann group indicated weight supporting plantar steps, but no forelimb-hind limb coordination. In contrast, the average recovery score in the control group was very low, showing only two joints of hind limbs that had extensive movement. Re-transection of the grafts at their mid-point in the M-Schwann group was performed 6 weeks after transplantation, which completely abolished the recovered hind limb function, and no significant recovery was observed even 4 weeks after re-transection (Kamada et al., 2005). These results exclude the possibility that transplanted cells enhanced the activity of a locomotor pattern generator in the spinal cord, but rather emphasize that axonal regeneration induced by transplanted M-Schwanns contributed to functional recovery.

Induction of functional neuronal cells from MSCs

Recently, we established a method to systematically and specifically induce neuronal cells from MSCs (Fig. 3). Highly efficient and specific induction of post-mitotic functional neuronal cells, without glial differentiation, can be achieved by gene transfer of Notch intracellular domain (NICD) followed by the administration of a certain combination of trophic factors (Dezawa et al., 2004) (Fig. 2).
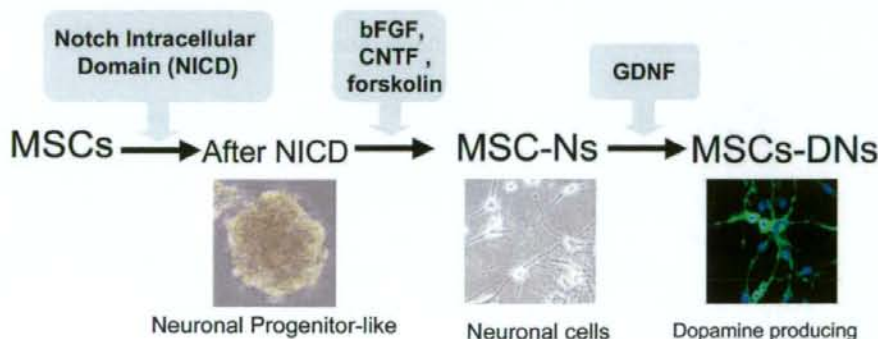

Notch Intracellular Domain (NICD) **bFGF, CNTF, forskolin** **GDNF**

MSCs → After NICD → MSC-Ns → MSCs-DNs

Neuronal Progenitor-like Neuronal cells Dopamine producing

Fig. 3. Induction of neuronal cells from MSCs. After introduction of Notch intracellular domain (NICD), MSCs change their property resembling neuronal progenitor cells. These cells are expanded in lower cell density and administrated with bFGF, CNTF and forskolin. After such treatment, MSC-Ns exhibited neuronal property such as action potential and neuronal marker expression. GDNF treatment differentiates MSC-Ns into dopamine producing cells (MSC-DNs) that is applicable to Parkinson's disease model.

The Notch signaling pathway has been known to influence on cell fate determination during development, and to maintain a pool of uncommitted precursors to the terminal specification of cells (Lundkvist and Lendahl, 2001). In the neural development, Notch is known to be one of differentiation factors for glial development, and in fact, a series of studies have shown that when Notch signaling is activated, astrocytes and Schwann cells differentiate from neural stem cells (NSCs) and neural crest stem cells, respectively. Initially, we expected that MSCs would shift from mesenchymal to Schwann cell characteristics by Notch introduction when combined with administration of trophic factors related to neural development. After such treatment, however, it was very surprising to see a small population of neuron-like cells induced in the final product. We repeated the experiment, modified and finally established neuronal induction system from MSCs as shown below.

The mouse Notch1 intracellular domain (NICD) cDNA was subcloned into a pCI-neo expression vector, and transfected into MSCs with lipofection followed by selection. After NICD introduction, MSCs acquired neuronal progenitor cell (NPCs) property (Fig. 3). In fact, they expressed markers related to neural stem cells and/or NPCs, and demonstrated sphere formation in the free-floating culture system. The morphology of NICD introduced cells do not differ from naive MSCs, but when they were expanded and then supplied with trophic factors (bFGF, FSK and ciliary neurotrophic factor (CNTF)) for several days, they changed their morphology drastically, extended neurite-like processes and differentiated into post-mitotic neuronal cells in efficiency of approximately 96% (MSC-Ns) (Fig. 3). These cells were immunopositive for neuronal markers, including MAP-2ab, neurofilament and ß-tubulin class III, and action potential was recorded in some of MSC-Ns in the patch clamp experiment. The outstanding character of MSC-Ns is that they are devoid of glial development in the final population. In fact, few positive cells either to GFAP (marker for astrocytes), and galactocerebroside and O4 (markers for oligodendrocytes) were detected in MSC-Ns. We then estimated whether MSC-Ns integrated into host brains to contribute to functional recovery in neurodegenerative disease models.

MSC-Ns contribute to functional recovery in stroke model

MSC-Ns were transplanted into the infarction area in middle cerebral artery occlusion (MCAO) rat model. Seven days after the occlusion, a total of 50,000 MSC-Ns were directly injected to the infracted area. The transplanted rat showed significant recovery in Beam balance (vestibulomotor function), Limb placing (sensorimotor function) and Morris water maze (cognitive function) test (p<0.01). Histologically, GFP-labeled transplanted cells migrated from the injection site into the ischemic boundary area, expressed neuronal markers of neurofilaments, MAP-2ab and ß-tubulin class

III, integrated into the hippocampus and cortex and extended processes. Most of the transplanted cells were neuronal marker-positive cells, while only a small number of cells (approximately 1%) were positive for GFAP. These results show that induced neuronal cells are effective in the amelioration of rat brain ischemic injury model.

Induction of dopamine producing cells and their application for Parkinson's disease

For Parkinson's disease, transplantation of dopaminergic neurons is believed to be effective (Kawasaki et al., 2000). However, cells committed positive for TH, a marker for dopaminergic neurons, accounted for lower ratios (~3%) in MSC-Ns initially (Dezawa et al., 2004). As glial-cell line derived neurotorphic factor (GDNF) is known to promote the generation and development of midbrain dopaminergic neurons (Akerud et al., 1999), GDNF was administered to MSC-Ns to increase the proportion of cells immunopositive for TH (Fig. 3). This treatment was effective, and approximately 40% of MSC-Ns became TH-positive (MSC-DNs). Importantly, the dopamine release upon depolarization in vitro was confirmed by HPLC, showing that MSC-DNs actually produced and released dopamine to the culture media in response to high K^+ depolarizing stimuli. These results indicate that functional dopamine producing neuronal cells can effectively be induced from MSCs (Dezawa et al., 2004) (Fig. 3).

Rat MSC-DNs were transplanted into the striatum of Parkinson's disease model rat induced by 6-hydroxy dopamine (6-OHDA). In these model rats, apomorphine injection induces abnormal rotational behavior, which is generally used as an indicator of Parkinson's disease symptoms in animal models. Rats grafted with MSC-DNs demonstrated substantial recovery from rotation behavior up to 10 weeks (Dezawa et al., 2004). In addition to rotational behavior, non-pharmacological behavior tests, adjusting step and paw-reaching tests demonstrated the significant improvement in behavior in both experiments. GFP-labeled transplanted cells integrated into the host brain and expressed marker of neurofilaments, TH and dopamine transporter (DAT) in the striatum, while few cells were positive for GFAP and O4, consistent with in vitro data. The recovery in production and release of dopamine after transplantation was also confirmed in the HPLC analysis of brain slice culture. Animals grafted were followed up to 16 weeks and there was no tumor formation observed in the brain.

Human MSC-DNs were similarly transplanted into the striatum of Parkinson's model rats under the control of immunosuppressant FK 506, and rotational behavior was recorded at four weeks after cell transplantation. Grafting resulted in significant improvement in rotational behavior as well (Dezawa et al., 2004).

Above results demonstrated that functional mature neurons with an ability to produce and release neurotransmitters are able to induce from rodent and

human MSCs. Recently, we reproduced the above system in cynomolgus monkey MSCs, are evaluating the efficiency and safety of MSC-DNs auto-transplantation in monkey Parkinson's disease model.

Induction of skeletal muscle cells from MSCs

During the experiment of neural induction, the order of treatment was reversed in order to perform the control experiment (Fig. 2). However, this event accidentally demonstrated the induction of skeletal muscle cells. The induction experiment was repeated, upgraded, and finally a new method to systematically and efficiently induce skeletal muscle lineage cells with high purity from large population of MSCs was established (Dezawa et al., 2005).

Human and rat MSCs were firstly treated with trophic factors of bFGF, FSK, PDGF and neuregulin, followed by transfection with a NICD expression plasmid by lipofection and selection, and allowed to recover to 100% confluency. At this stage, the majority of MSCs developed to mononucleated myogenic cells expressing skeletal muscle cell-specific marker, MyoD. Cells were then supplied with differentiation medium (2% horse serum or ITS Insulin-Transferrin-Selenite-serum free medium, both of which are known to promote differentiation of myoblasts to myotubes) (Yoshida et al., 1998) (Fig. 4). After treatment, MSC-derived muscle lineage cells (MSC-Ms) were obtained. This final population contained 3 kinds of muscle-lineage cells; (1) post-mitotic multinucleated myotubes expressing Myf6/MRF4 (a marker for mature skeletal muscle) and contractile proteins (2) mononucleated myoblasts: expressing MyoD, and (3) satellite-like cells: positive for Pax7, marker for muscle satellite cells (Seale et al., 2000).

Application to muscle degeneration models

To estimate how workable these induced muscle lineage cells are in the repair of degenerated muscles, human MSC-Ms were transplanted into immuno-suppressed rats whose gastrocnemius muscles were damaged with cardiotoxin pretreatment (Fukada et al., 2002). Cells were transplanted by local injection into degenerated muscles. Two weeks after transplantation, GFP-labeled transplanted cells incorporated into newly formed immature myofibers, exhibited centrally located nuclei in treated animals. Four weeks after transplantation, GFP-positive myofibers exhibited mature characteristics with peripheral nuclei just beneath the plasma membrane. Functional differentiation of grafted human cells was also confirmed by the detection of human dystrophin in GFP-labeled myofibers (Dezawa et al., 2005).

MSC-Ms contained cells those developed into satellite-like cells in the host muscle. In general, muscle satellite cells are known to contribute to regenerating myofiber formation upon muscle damage (Bischoff, 1994). Therefore, we tested whether transplanted satellite-like cells were able to contribute to muscle regeneration as satellite cells in vivo. Four weeks after transplantation of human MSC-Ms, cardiotoxin was re-administered into the same muscles without additional transplantation. Two weeks after the second cardiotoxin treatment (6 weeks after initial transplantation), many regenerating GFP-positive myofibers with centrally-located nuclei were observed. This implies that, upon transplantation of MSC-Ms to muscles of patients, those retained as satellite cells are to be able to continue to contribute to future muscle regeneration (Dezawa et al., 2005).

Compared to the various muscle stem cell systems that have been reported, this system offers several important advantages. Since our induction system does not depend on a rare stem cell population, but utilizes the general population of adherent MSCs, which are easily isolated and expanded, functional skeletal muscle cells can be obtained within a reasonable time course on a therapeutic scale. In the case of MSCs derived from inherited muscle dystrophy patients, genetic manipulation is possible after the isolation and expansion of MSCs. Moreover, transplantation of MSC-derived cells should encounter fewer ethical problems.
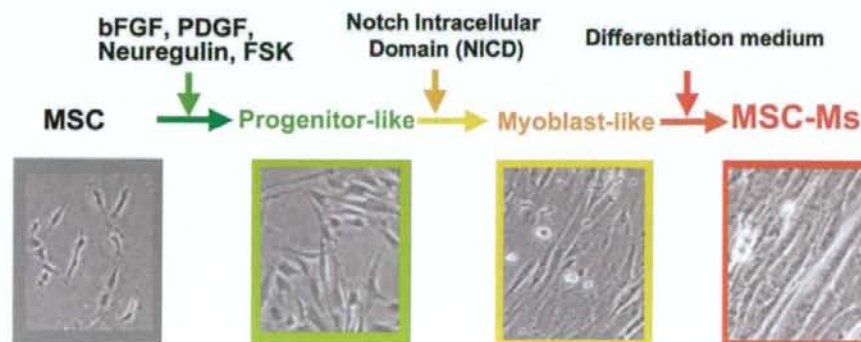

bFGF, PDGF, Neuregulin, FSK   **Notch Intracellular Domain (NICD)**   **Differentiation medium**

MSC → **Progenitor-like** → **Myoblast-like** → **MSC-Ms**

Fig. 4. Induction of skeletal muscle lineage cells from MSCs. MSCs generate Pax7-positive precursor cells after trophic factor stimulation and, after NICD transfection, induce MyoD- and myogenin-positive myoblasts. Myoblasts fuse to form multinucleated myotubes by differentiation medium, expressing the marker of maturity, MRF4/Myf6.

General conclusions

While ES cells and tissue stem cells have great potential, MSCs also provide hopeful possibilities for clinical application, since they can be efficiently expanded in vitro and we could acquire a therapeutic scale of induced cells. In addition, transplantation of MSC-derived cells should pose fewer ethical problems by preventing stem cell controversy, since bone marrow transplantation has already been widely performed. As MSCs are easily obtained from patients or marrow banks, autologous transplantation of induced cells or transplantation of induced cells with the same HLA subtype from a healthy donor may minimize the risks of rejection (Fig. 5). Needless to say, bone marrow should at least be 'normal and healthy' for transplantation. Particularly, in the case of autologous transplantation in muscular dystrophy, the replenishment of the normal gene is necessary for the use of patient's MSCs. In such case, usage of MSCs from a healthy donor with the same HLA subtype may be a more realistic solvent.

Although we showed the high ratio and specific induction of Schwann cells, neurons and skeletal muscle cells, we still have to solve the following problems (Dezawa et al., 2001, 2004, 2005). Although there have been so far few reports referring to tumor formation after transplantation of untreated MSCs, further studies are needed to ensure safety, tumor formation and efficacy of manipulated MSCs over a long-term period using higher mammals such as primates. Secondly, as the potential of differentiation would differ by age, individual, race, and sexes, each of these must be investigated in the future. Third, MSCs have been shown to be heterogeneous in terms of growth kinetics, morphology, phenotype, and plasticity. With the development of specific markers and detailed characterization of heterogeneous general adherent MSCs, their properties and plasticity can be studied and defined with more certainty. Fourth, the use of fetus bovine serum in our induction system is problematic due to the risk of infections and BSE. Fortunately, as we already confirmed that human serum is more appropriate to the differentiation of human MSCs than fetus bovine serum (unpublished data), this system is able to provide patient's MSC-derived cells using the patient's own serum.

Notch–Hes signaling are known to inhibit neuronal and myogenic differentiation in conventional development (Lundkvist and Lendahl, 2001). However, in our system, NICD introduction accelerated the induction of neuronal and skeletal muscle cells from MSCs. Although our results appear inconsistent with previous work, they do not refute the known role of Notch-Hes signals during development. In the previous report, JAK/STAT inhibitor administration and constitutive active STAT1/3 transfection showed that down regulation of STATs was tightly associated with NICD-mediated neuronal induction, whereas Hes, down stream of Notch, was not involved in the induction event (Dezawa et al., 2004). Skeletal muscle induction was also revealed to be independent of Hes1/5. Thus, our results suggest the distinct cellular responses to Notch signals; for example, the repertoire of second messengers and active factors may well be different between conventional neural stem cells and/or neural progenitor cells and MSCs. It might be possible that an unknown signaling pathway downstream of Notch may be involved in these events, and thus further studies are needed to identify the factor involved in this

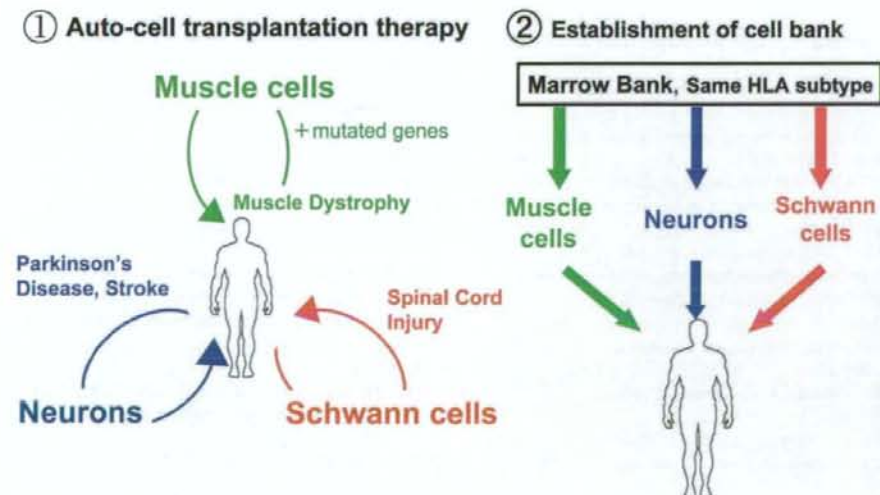**① Auto-cell transplantation therapy**

② Establishment of cell bank

Fig. 5. Schematic diagram of "auto-cell transplantation system" and "cell bank system" using patient's or donor-derived MSCs. Neurons, Schwann cells, and skeletal muscle cells induced from patient's MSCs or marrow bank-derived MSCs that exhibit same HLA subtype are transplanted back to the patient. Particularly, a self-regenerative system avoids ethical issues and immuno-rejection.

phenomenon.

Since MSCs can be obtained from patients, it is possible to establish "auto-cell transplantation therapy" using MSCs (Fig. 5). Implantation of naive cells would be expected for trophic and immune modulatroy effects that may contribute to the functional recovery in a way. In the incipient stage, such protective treatment will be effective to prevent the progressive loss of damaged cells, however, in the advanced stage, cell replacement will provide the basis for the development of potentially powerful therapeutic strategies. Importantly, little can be expected of the spontaneous differentiation of MSCs. For the purpose of cell replenishment, strategic and systematic induction of MSCs should be necessary. To realize this ideal, it is necessary to develop the regulatory system of differentiating MSCs into cells with a purpose. Our method would be one of the possible ways to regulate MSC transdifferentiation into functional Schwann cells, neurons and skeletal muscle cells, which will be applicable to neurodegenerative and muscle degenerative diseases.

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

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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

A N 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 barrier 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

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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
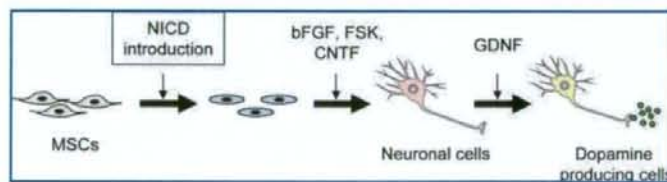

FIG. 1. Induction system of dopaminergic neurons. Bone marrow stromal cells (BMSCs) were transfected with the pCI-Notch intracellular domain (NICD) plasmid, selected using G418. Treatment with trophic factors basic fibroblast growth factor (bFGF), forskolin (FSK), and ciliary neurotrophic factor (CNTF) leads BMSCs to differentiate 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
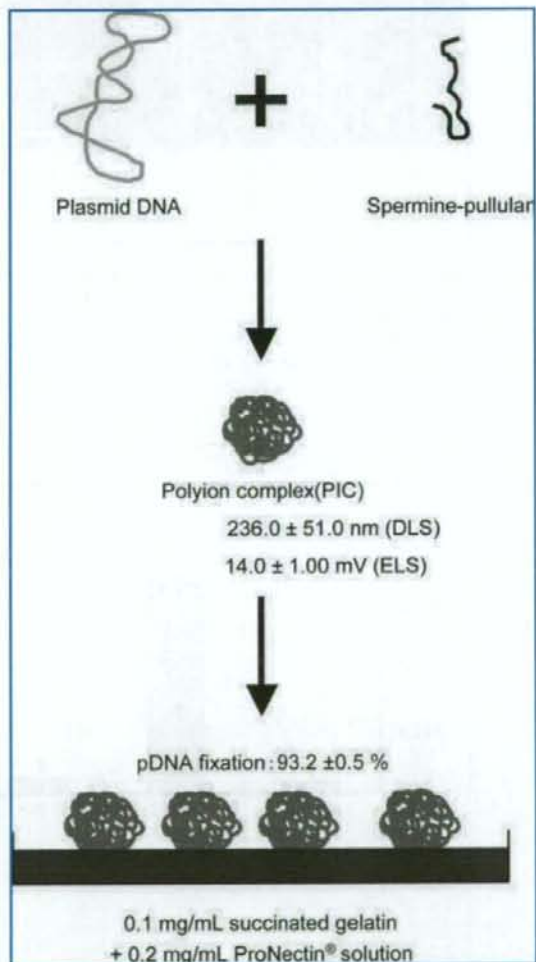

FIG. 2. Schematic illustration of the spermine-pullulan-mediated reverse transfection. The plasmid DNA was intermingled with the spermine-pullulan to generate the polyion complex (PIC). The PIC was fixed on the surface of the culture dish, which had been coated with succinated gelatin and Pronectin before 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).
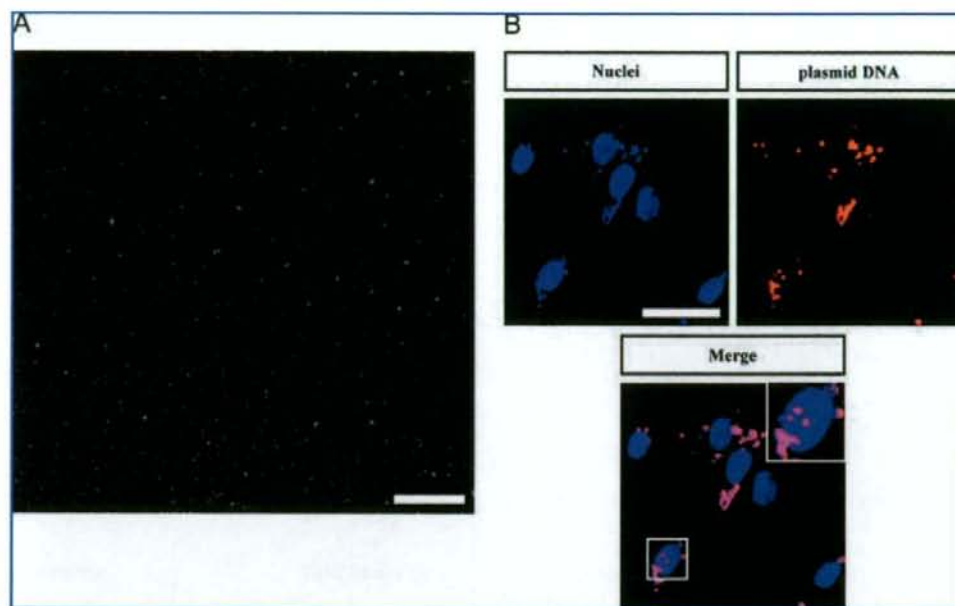
FIG. 3. (A) Confocal laser microscopic image of the surface of culture dishes fixed with the polyion complex (PIC) containing Cy5-labeled plasmid DNA. Plasmid DNA was visualized using Cy5 fluorescence. (B) Confocal laser microscopic images of monkey bone marrow stromal cells transfected with the Cy5-labeled 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.
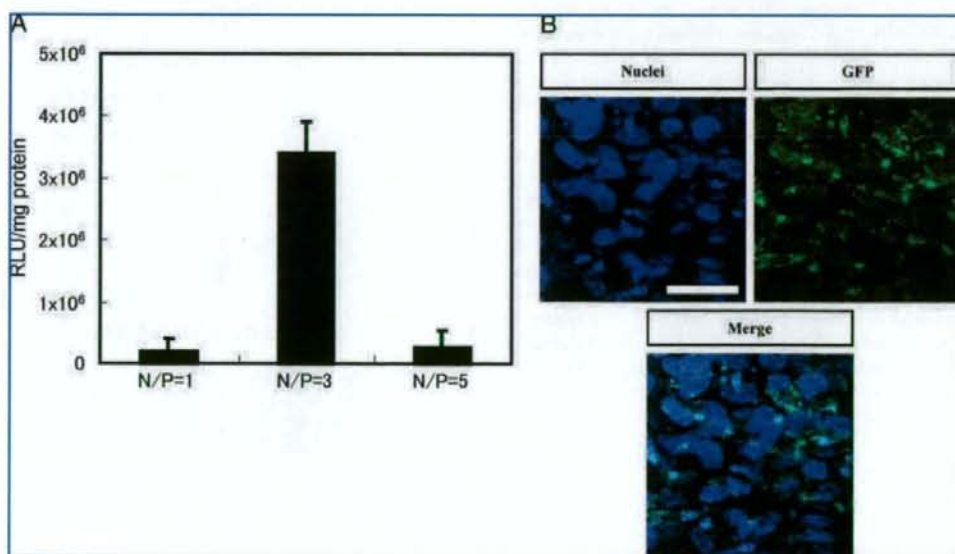

FIG. 4. (A) Luciferase assay for the determination of N/P ratio. Three conditions for the nitrogen number of the spermine-pullulan (N) per the phosphorus number of plasmid DNA (P) (the N/P ratio), 1, 3, and 5, were employed for the spermine-pullulan-mediated reverse transfection with pGL3 vector. Substantial 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.

5

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.
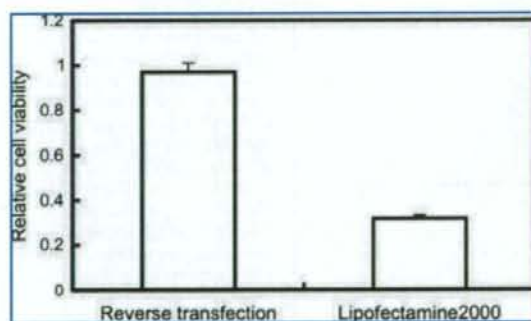

FIG. 5. Cell viability of transfected monkey bone marrow stromal cells (BMSCs) in which the spermine-pullulan-mediated reverse transfection (left columns) or Lipofectamine 2000–mediated transfection (right columns) delivered the pCI-Notch intracellular domain (NICD) plasmid 3 days before the evaluation. MSCs transfected using the spermine-pullulan-mediated reverse transfection method exhibited higher cell viability ($97.0 \pm 4.1\%$) than that of Lipofectamine 2000 ($31.6 \pm 1.4\%$).
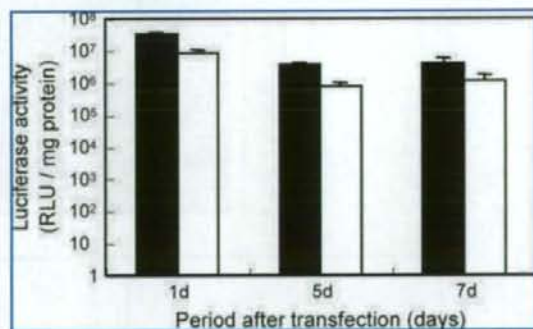

FIG. 6. Periodical analysis of luciferase activity in monkey bone marrow stromal cells (BMSCs) transfected with pGL3 plasmid vector using the Lipofectamine 2000–mediated regular transfection (closed columns) or the spermine-pullulan-mediated reverse transfection technique (open columns) 1, 5, and 7 days 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.
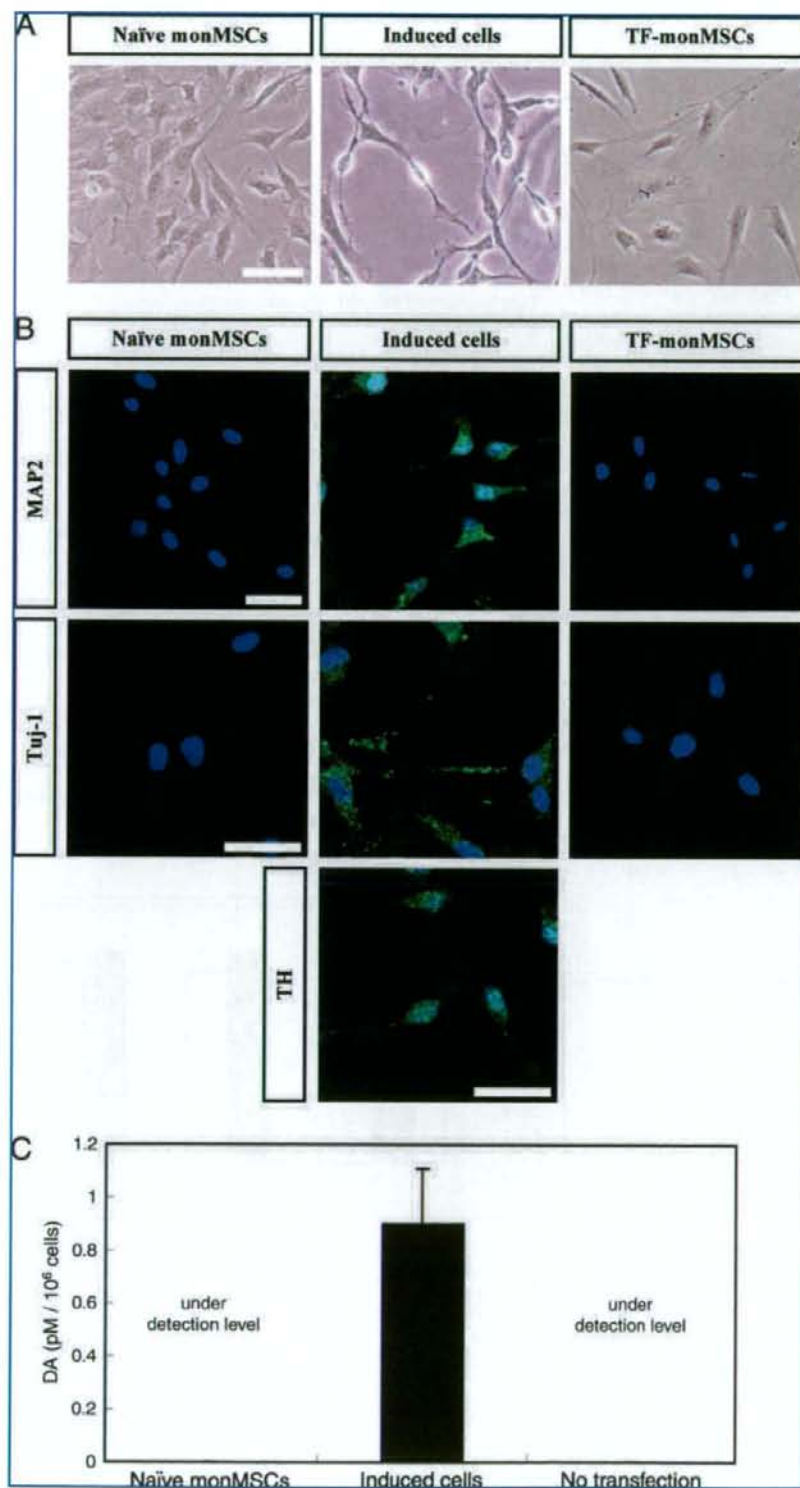
FIG. 7. Morphological and functional characterization of naïve monkey bone marrow stromal cells (monBMSCs) induced neuronal cells and trophic factor–treated monMSCs without the reverse transfection of pCI-Notch intracellular domain (NICD; TF-monMSCs, see text). (A) Phase contrast images. Induced neuronal 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: $100\,\mu m$.

Low toxicity of spermine-pullulan-mediated transfection

Next, we measured the viability of cells transfected with plasmid DNA. Figure 5 showed that only $31.6 \pm 1.4\%$ 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, $97.0 \pm 4.1\%$ 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] Immu-

nocytochemistry 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
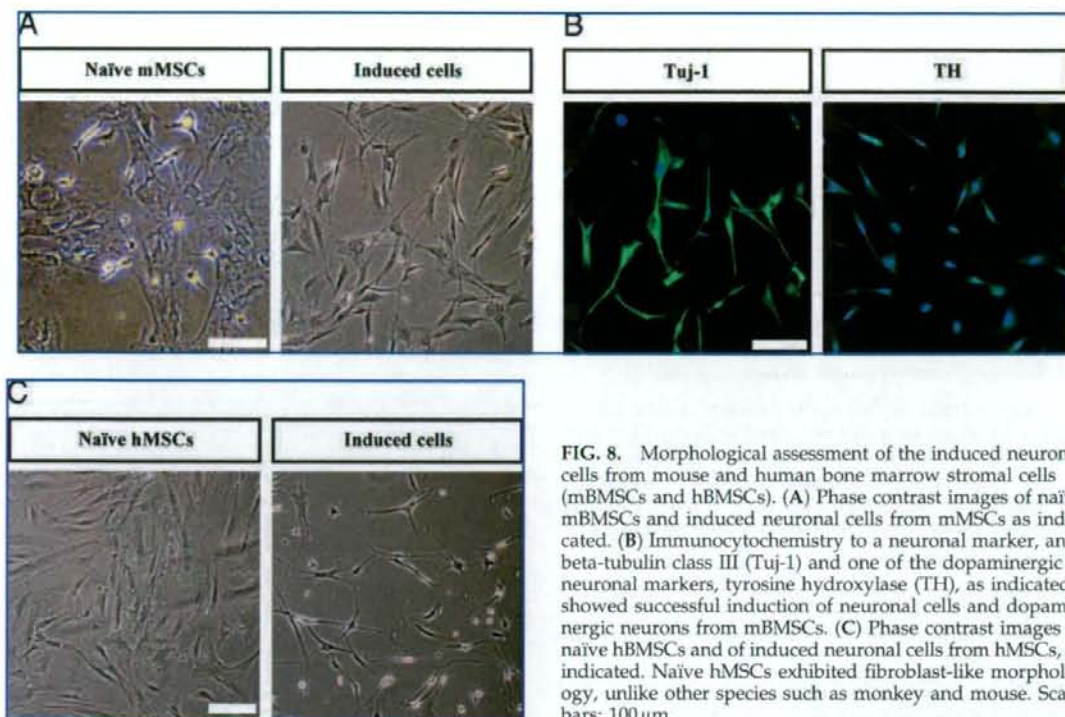
FIG. 8. Morphological assessment of the induced neuronal cells from mouse and human bone marrow stromal cells (mBMSCs and hBMSCs). (A) Phase contrast images of naïve mBMSCs and induced neuronal cells from mMSCs as indicated. (B) Immunocytochemistry to a neuronal marker, anti-beta-tubulin class III (Tuj-1) 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 identi-

cal 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.

Acknowledgments

Disclosure Statement

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