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Review

Induction system of neural and muscle lineage cells from bone marrow stromal cells; a new strategy for tissue reconstruction in degenerative diseases

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Summary. Since bone marrow stromal cells (MSCs) are easily accessible both from healthy donors and patients, and can be expanded on a therapeutic scale, they have attracted attention for cell-based therapy. MSCs contribute to the protection of host tissue after transplantation by Immune modulation and trophic effect. They also have an ability to differentiate into other cell kinds that will replenish lost cells in the degenerated tissue. This review discusses the potential of MSCs for tissue reconstruction in neuro- and muscle-degenerative diseases and their differentiation capacity into functional cells.

Key words: Mesenchymal stem cells, Muscle dystrphy, Parkinson's disease, Spinal cord injury, Stroke

Introduction

Bone marrow contains a category of nonhematopoietic cells that can be cultivated and expanded in vitro as plastic adherent cells. These cells normally provide structural and functional support for hematopoiesis, and are called bone marrow stromal cells, mesenchymal stem cells or bone marrow stromal stem cells, but an uniform term for these cells is not fixed yet. In this review, these cells are called bone marrow stromal cells (MSCs). Since they exhibit diverse characteristics and consist of heterogeneous population, their true nature is not fully understood. The majority of MSC population express mesenchymal markers, such as CD29 (beta1-integrin), CD90 (Thy-1), CD54 (ICAM-1), CD44 (H-CAM), CD71 (transferrin receptor), CD105 (SH2), SH3, Stro-1, and CD13, but a small number of cells are positive for hematopoietic surface markers, such as CD34, CD3, CD117 (c-kit) (Pittenger et al., 1999, 2000). They behave like stem cells, while their stem property is still subject for debate.

They are easily accessible through the aspiration of the bone marrow, can be isolated from patients, and can be expanded in a large scale, both from healthy donors and patients. For example, 20-100 ml of bone marrow aspirate yields $1x10^7$ of MSCs within several weeks, which provides a plentiful number of cells.

Recently, MSCs have attracted attention mainly from two aspects. One is that they contribute to the protection of host tissue after transplantation, mainly by immune modulation and trophic effect. As MSCs originally support hematopoietic cells in the bone marrow, they produce various kinds of cytokines and trophic factors. This nature is beneficial to tissue protection, controlling apoptosis and neovascularization. In fact, when naive MSCs are transplanted to neuro-traumatic or -degeneration models, such as spinal cord injury, strokes and experimental autoimmune encephalomyelitis (EAE), or to myocardiac infarction, they migrate into the damaged site, protect tissues and partly contribute to the functional recovery (Chopp et al., 2000, 2008; Lu et al., 2001; Ohta et al., 2004, 2007; Ohnishi et al., 2007; Qu et al., 2007, 2008; Zhang et al,, 2005, 2006). The other reason is that they have an ability to differentiate into other cell kinds that will replenish lost cells in the degenerated tissue.

MSCs and immune system

MSCs have been suggested to be 'immune-privileged" because of their low expression of major histocompatibility complex (MHC) class I and no expression of class II (Uccelli et al., 2006). This characteristic of MHCs is expected to diminish the

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reaction of graft rejection. There are several hopeful reports regarding the transplantation of MSCs (Liechty et al., 2000; Tse et al., 2003; Niemeyer et al., 2006; Fibbe et al., 2007; Wei et al,, 2008). Tse et al. reported that T-cells failed to detect MSCs (Tse et al., 2003), Liechty et al. showed that transplantation of human MSCs into sheep gave no specific rejection against the grafted cells (Liechty et al., 2000), and Wei et al. demonstrated that only the CD34-negative fraction derived from human bone marrow survived after grafting into rat intervertebral discs with high expression of Fas-ligand, which has been implemented in the reduction of allogenic rejection independent of apoptotic induction (Wei et al., 2008). However, there are some studies objecting to this idea. Grafted MSCs into allogeneic, MHC-mismatched mice resulted in considerable rejection (Eliopoulos et al., 2005; Nauta et al., 2006). Antigen presentation by MSCs under specific stimulation such as interferon-γ (IFN-γ) pretreatment was also reported (Chan et al., 2006). In our study, human MSCs implanted into the rat sciatic nerve tended to be rejected even with mild immunosuppression (Shimizu et al., 2007). Thus, more attention should be paid to evaluating the MSC's immune-privilege.

MSCs are known to modify their circumstances to suppress the immunoreaction. MSCs bring cell division arrest to T-cells (Glennie et al., 2005), B-cells (Corcione et al., 2006), natural killer (NK) cells (Spaggiari et al., 2006), and dendritic cells (DCs) (Ramasamy et al., 2007). The cell division arrest on T-cells by MSCs is caused by inhibition of cyclin D2 expression, thus cell cycle is arrested in the G0-G1 phase (Glennie et al., 2005). Crosstalk of MSCs and immune cells are also important for MSCs to exhibit the inhibition effect on proliferation of immune cells. T-cells and NK cells secrete IFN-γ (Krampera et al., 2006) to stimulate MSCs to produce indoleamine 2,3-dioxygenase (IDO), which inhibit proliferation of T-cells and NK cells (Krampera et al., 2006). Monocyte release IL-1ß so that MSCs are sensitized to secrete transforming growth factor-ß1 (TGF-ß1) (Groh et al., 2005). Neither system utilizes cell-cell contact mechanism, indicating that MSC-regulated inhibition of immune cell proliferation is dependent on cell-to-cell communication via soluble factors.

MSCs have been reported to affect other immunological reactions: secretion of cytokines and cytotoxicity of T-cells (Krampera et al., 2003; Rasmusson et al., 2003; Aggarwal and Pittenger, 2005; Zappia et al., 2005) and NK-cells (Spaggiari et al., 2006), maturation and antibody secretion of B-cells (Corcione et al., 2006), and maturation, antigen presentation, and activation of DCs (Ramasamy, Fazekasova et al., 2007). This mediation of immunoreactions by MSCs is considered to be regulated by secretion of molecules by MSCs, such as hepatocyte growth factor and TGF-ß1 (Di Nicola et al., 2002), IDO (Meisel et al., 2004), nitric oxide (Sato et al., 2007), and prostagrandin E2 (Aggarwal and Pittenger, 2005), demonstrated by in vitro studies. Further studies are needed to elucidate MSC's function on in vivo immunomodulation through specific molecules.

MSC's function on inhibition of immune reactions has been applied for treatment of autoimmune diseases, or diseases caused by immunological dysfunction. Graft-versus-host disease (GvHD) is one of the targets for MSC to play a critical role on immunomodulation (Le Blanc et al., 2004; Ringden et al., 2006). Co-infusion of MSCs, in addition to hematopoietic stem cells, both derived from sibling donor bone marrow, has been demonstrated to decrease the sporadic rate and severity of GvHD (Lazarus et al., 2005). This strategy is now under clinical trials, and the results from Phase I study proved the feasibility and safety of the grafting of cultured MSCs (Lazarus et al., 1995). The other study showed the similar effect on the treatment of GvHD using co-infusion technique, in which donor bone marrow was transplanted with host MSCs (Aksu et al., 2008). For other diseases, including rheumatoid arthritis, systemic lupus erythematosus and multiple sclerosis, MSCs have been applied in animal models, but the mechanism of improvement of pathological condition caused by MSC infusion is mostly unknown.

Tissue repair by MSCs implantation

Infused MSCs are reported to migrate to the vast majority of organs where these cells integrate and differentiate into tissue specific cells in the irradiated recipient (Devine et al., 2003). Also, MSCs are known to migrate into the injured site (Chen et al., 2001; Ohta et al., 2004), suggesting that they might have the property to recognize the environmental cues to make them chemotaxis to the injury tissues. Recent studies have shown the mechanisms of this chemotactic property of MSCs: MSCs have been shown to have receptors related to chemotaxis, such as chemokine (C-X-C motif) receptor 4 (CXCR4) (Sordi et al., 2005). CXCR4 plays a critical role in homing of MSCs to the bone marrow, and this phenomenon is mediated by interaction of CXCR4 and its ligand, stromal-derived factor-1 (SDF-1) (Ji et al., 2004). Recent study also showed that MSCs express functional formyl peptide receptor (FPR), which also mediates chemotactic signaling, and MSCs are chemotactically migrated under the ligand-binding assay with intracellular calcium increase, mitogen-activated protein kinases activation and Akt activation (Kim et al., 2007). Toll-like receptors (TLRs) are known to be expressed in MSCs, which regulate proliferation and differentiation of MSCs (Pevsner-Fischer et al., 2007). Besides, MSCs can pass through the basement membrane by secreting metalloproteases (MMPs), in which MSCs are activated by cytokines, including IL-1ß, TGF-ß1, and tumor necrosis factor-a (Ries et al., 2007).

MSCs have been shown to exhibit a protecting effect on injured tissues, especially in the animal models of some neurological diseases, such as spinal cord injury

(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

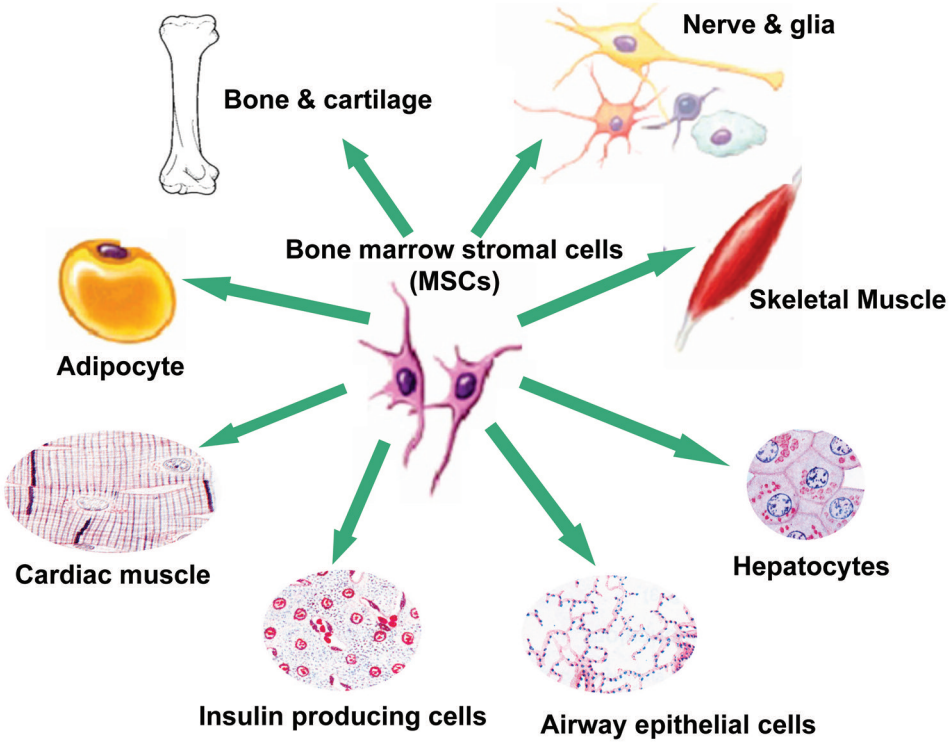

Fig. 1. Differentiation ability of MSCs into different kinds of cells.

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

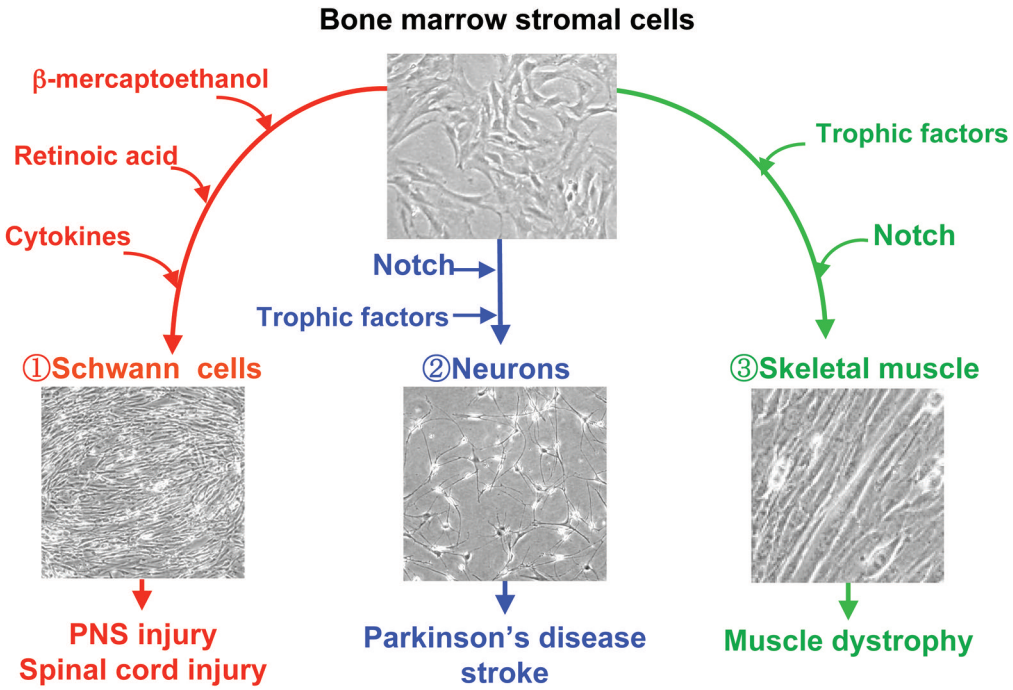

Fig. 2. Induction system of Schwann cells, neurons, and skeletal muscle cells in MSCs by combining trophic factor or cytokine treatment with Notch intracellular domain introduction. Induced cells are applicable to PNS and spinal cord injury models, Parkinson's disease and stroke models, and muscle dystrophy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

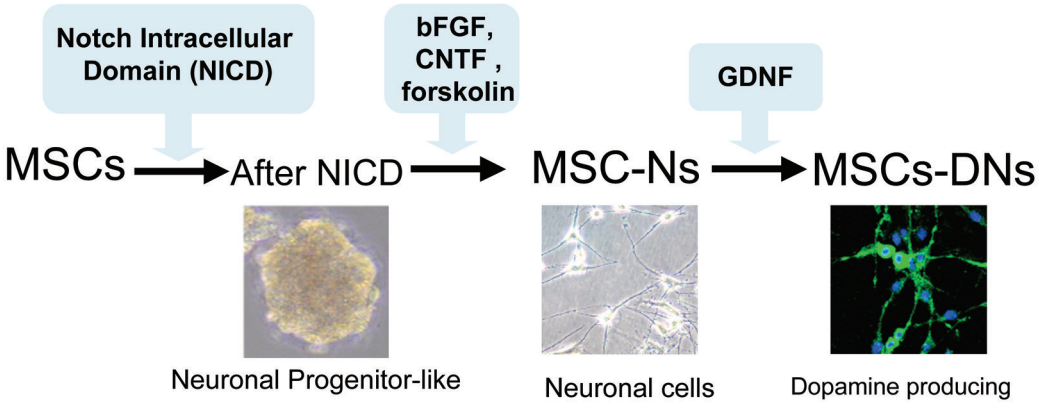

Fig. 3. Induction of neuronal cells from MSCs. After introduction of Notch intracellular domain (NICD), MSCs change their property resembling neuronal progenitor cells. These cells are expanded in lower cell density and administrated with bFGF, CNTF and forskolin. After such treatment, MSC-Ns exhibited 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.

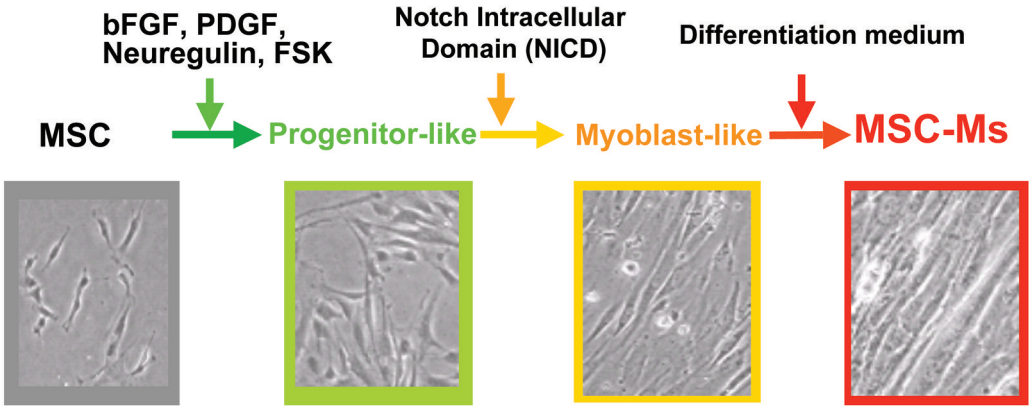

Fig. 4. Induction of skeletal muscle lineage cells from MSCs. MSCs generate Pax7-positive precursor cells after trophic factor stimulation and, after NICD transfection, induce MyoD- and myogenin-positive myoblasts. Myoblasts fuse to form multinucleated myotubes by differentiation medium, expressing the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

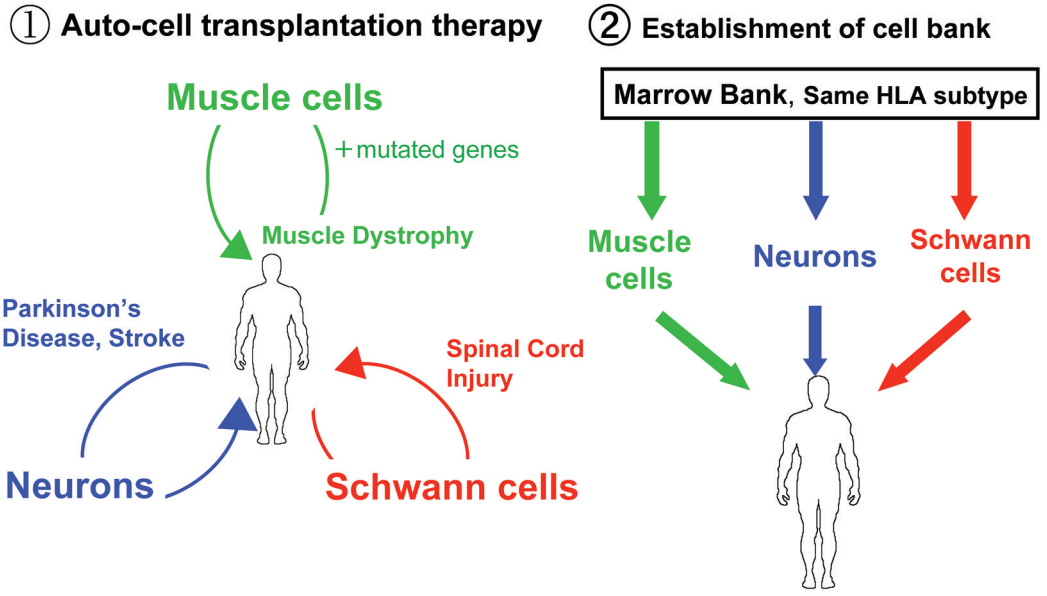

Fig. 5. Schematic diagram of "auto-cell transplantation system" and "cell bank system" using patient's or donor-derived MSCs. Neurons, Schwann cells, and skeletal muscle cells induced from patient's MSCs or marrow bank-derived MSCs that exhibit same HLA subtype are transplanted back to the patient. Particularly,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