Potential of Bone Marrow Stromal Cells in Applications for Neuro-Degenerative, Neuro-Traumatic and Muscle Degenerative Diseases

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Abstract: Cell transplantation is a promising strategy for the treatment of neurodegenerative and muscle degenerative diseases. Many kinds of cells, including embryonic stem cells and tissue stem cells, have been considered as candidates for transplantation therapy. Bone marrow stromal cells (MSCs) have great potential as therapeutic agents since they are easy to isolate and can be expanded from patients without serious ethical or technical problems. We discovered a new method for the highly efficient and specific induction of functional Schwann cells, neurons and skeletal muscle lineage cells from both rat and human MSCs. These induced cells were transplanted into animal models of neurotraumatic injuries, Parkinson's disease, stroke and muscle dystrophies, resulting in the successful integration of transplanted cells and an improvement in behavior of the transplanted animals. Here we focus on the respective potentials of MSC-derived cells and discuss the possibility of clinical application in degenerative diseases.

Key Words: Mesenchymal cell, transdifferentiation, regenerative medicine, cell therapy, transplantation, Schwann cells, neuronal differentiation, myogenic differentiation.

1. INTRODUCTION

Neurodegenerative and neuro-traumatic diseases such as Parkinson's disease, brain ischemia and spinal cord injury, and muscle degenerative diseases represented by muscle dystrophy, are responsible for a decline in neuronal and muscular function which often limit quality of life as well as life span. While solid organ transplantation such as liver, heart and kidney has already been performed on thousands of patients, the nervous systems and general muscle tissue have faced a great many limitations. Thus, it is hoped that effective therapeutic strategies will be developed. In the central nervous system (CNS), where neurons become post-mitotic after birth, cell transplantation is one of the hopeful strategies with a potential for treatment of such neural disorders. Although satellite cells are considered to be stem cells of muscle tissue [6], there is a need to establish cell therapies based on healthy donors since muscle dystrophies are inheritable diseases.

Recently, ES cells and tissue stem cells have aroused a great deal of interest among health professionals and basic scientists because of their potential for treating degenerative diseases. ES cells are known to differentiate into various kinds of cells including neural cells and skeletal muscle cells [53]. The neural differentiation of human ES cells has been studied recently, showing that neural progenitors could be obtained by spontaneous differentiation of ES cells using serum-free culture conditions [53]. More specialized neurons, such as tyrosine hydroxylase (TH)-positive dopaminergic neurons and telencephalic precursors are also reported to be inducible from ES cells [33, 72].

Tissue specific stem cells are identified in various tissues of more advanced developmental stages. Multipotent neural stem cells (NSCs) have been identified in developing and adult CNS tissues [9, 39, 54, 61, 73]. These cells have the ability to self-renew and the multipotent potential to differentiate into neurons, astrocytes and oligodendrocytes. In the name of neuronal cell replacement, transplantation of NSCs has been challenged in a wide range of animal models of diseases and injuries such as Parkinson's disease, Huntington's disease, stroke and spinal cord injury, and functional recovery has often been reported [27, 28, 35, 41, 46, 63]. As to muscle tissue, muscle stem cells and satellite cells isolated from adult and prenatal tissues [3, 21, 50, 68] and myogenic stem cells contained in the bone marrow [25, 64] are considered to be a source of stem cells, and there have been several attempts to ameliorate muscle degeneration by transplantation of these muscle stem cells [25]. Although ES cells and NSCs have great potential for cell replacement strategy, they face limitations inherent in procurement from fetal tissue, including problems of histocompatibility, inadequate tissue supply and ethical concerns.

Bone marrow contains a category of nonhematopoietic multipotent cells that can be cultivated *in vitro* as plastic adherent cells, namely bone marrow stromal cells (MSCs) [52]. MSCs are mesenchymal elements normally providing structural and functional support for hemopoiesis, 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 lack the hematopoietic surface markers such as CD34, CD3, CD117(c-kit) [48, 49]. They are easily accessible through the aspiration of the bone marrow, can be isolated from patients without touching ethical problems, and can easily be expanded in a large scale for autotransplantation. Thus, they are hopeful candidate for use in cell-based therapy.

According to a hierarchical paradigm, MSCs differentiate into mesenchymal lineage cells such as osteocytes,

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chondrocytes and adipocytes [52]. Chen *et al.* showed that cell shape, cytoskeletal tension and endogenous RhoA-Rock activities regulate the commitment of MSCs either to osteocytes and adipocytes [40]. Recently, however, unorthodox plasticity of MSCs have been described in that they have an ability to cross oligolineage boundaries which were previously thought to be uncrossable. In fact, various kinds of cells have been reported to be inducible from MSCs both *in vivo* and *in vitro*. The possibility of MSC plasticity and transdifferentiation was initially described in *in vivo* experiments, in that transplanted donor bone marrow-derived cells differentiated into glial cells in the recipient brain [42]. In the case of muscle, infusion of bone marrow cells integrated into host muscles and supported regeneration [25]. While these studies suggested plasticity of MSCs based on expression of donor marker and cell specific markers, clonality and functions of these transdifferentiated cells was not clearly estimated in some cases. Moreover, these phenomena have been suspected to partly rely on cell fusion or spontaneous trans-differentiation with extremely rare frequency [2, 65].

Apart from *in vivo* experiment, there have been several trials *in vitro* to induce MSCs into purposive cells. Makino *et al.* showed that rhythmically contracting cardiomyocytes with cardiac muscle markers and electrophysiological characters could be induced from MSCs [38]. In the case of neuronal induction, several reports demonstrated that MSCs can differentiate into neuroectodermal characteristics [34, 57] and into neuron-like cells [34]. Other than cardiac muscle cells and neuronal cells, hepatocytes, insulin-producing cells and airway epithelial cells are also reported to be inducible

from MSCs, while some of these reports contain lower induction efficiency [11, 70, 71]. Accordingly, the potential of MSCs to transdifferentiate from mesenchymal lineages to other lineages is now of great interest. While they offer great potential for cell transplantation therapy, their practical application to human degenerative diseases is dependent on the ability to control their differentiation into specific functional cells with high efficiency and purity.

Recently, we have developed a method to systematically induce Schwann cells, neurons and skeletal muscle cells from human and rat MSCs on a therapeutic scale [17-19] (Fig. 1). In this review, we focus on the potentials, benefits and drawbacks of MSCs and discuss the possibility of clinical application in neuro-degenerative, neuro-traumatic and muscle-degenerative diseases. The usefulness of ES cells, NSCs and other kinds of stem cells have been reviewed else where [53].

2. SCHWANN CELL AND AXONAL REGENERATION

One kind of glial cell, namely Schwann cells, which reside in the peripheral nervous system (PNS), is known to support axonal elongation by providing various kinds of trophic factors, cytokines, cell adhesion molecules and extracellular matrix, and finally to reconstruct myelin of regenerated axons [15, 22, 23]. In PNS, damaged nerves are known to successfully regenerate although occasionally they do not form functional connections due to a lack of topographic connections. In contrast, it has been assumed that CNS had little capacity to regenerate neurites unless they are provided with adequate circumstances [62]. A

Fig. (1). Schematic diagram of induction system from human and rat MSCs. Schwann cells could be induced by treatment with beta-mercaptoethanol (BME), retinoic acid (RA) followed by trophic factor administration of bFGF, forskolin (FSK), PDGF and neuregulin. After treatment with BME and RA, cell morphology differs from original untreated MSCs. In the final step, MSCs became similar to Schwann cells, and express Schwann cell markers of p75. Neurons are induced by Notch intracellular domain (NICD) gene transfer followed by trophic factor administration of bFGF, FSK and CNTF. The final population is consisted mostly of neurons immunopositive to neuronal markers such as neurofilament. Skeletal muscle lineage cells could be obtained by trophic factor treatment of bFGF, FSK, PDGF and neuregulin, followed by NICD gene transfer. In this step, MSCs express some of skeletal muscle markers of MyoD and myogenin, and their morphology became similar to myoblasts. Furthermore, MSC supernatant treatment generates multinucleated myotubes.

feasible strategy for augmenting CNS axonal regeneration was examined and found that nerve regeneration was promoted by adopting the favorable properties exhibited in injured PNS; specifically, provision of trophic factors and grafting of Schwann cells. Moreover, many CNS axons are myelinated by oligodendrocytes. Myelinating cells, either of Schwann cell or oligodendrocyte origin, mediate the spacing of sodium channel clusters at the nodes of Ranvier to enable saltatory conduction, which is a prerequisite for normal neuronal activity and function. Therefore, even if the CNS can elongate its axons after axotomy, remyelination of regenerated axons is indispensable for the re-establishment of CNS function. Although Schwann cells originally myelinate PNS axons, they can also remyelinate CNS axons when transplanted. From 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. In fact, a series of experiments have demonstrated that transplanted Schwann cells can delay nerve cell death and prevent axonal degeneration [16, 69], and Schwann cells included in a transplanted peripheral nerve segment or cultured Schwann cells, supplied into the damaged optic nerve and spinal cord, can promote regeneration of nerve fibers by distances of several centimeters.

Although Schwann cells have a strong ability to induce nerve regeneration both in PNS and CNS, it is difficult clinically to obtain sufficient amount of cells. To cultivate Schwann cells for autotransplantation in human, another peripheral nerve must be sacrificed. There also remain technical difficulties in harvesting and expanding Schwann cells. Accordingly, it would be more desirable to establish cells of Schwann cell characteristic from sources other than PNS which are easy to access, capable of rapid expansion, amenable to survival and integration into host tissue and of eliciting axonal regeneration and re-myelination.

2.1. Induction of Schwann Cells from MSCs

Recent reports have described the use of MSCs for transplantation in models of PNS injury. Cuevas *et al.* described the migration and integration of MSCs following the injection into the site of a sciatic nerve repair [14]. Terenghi *et al.* treated MSCs with glial growth factor, a powerful Schwann cell mitogen, to induce them to GFAP and S-100 positive cells and recognized a small population of Schwann cell-like cells within the treated MSCs [67]. We have reported that MSCs can be transdifferentiated into cells with Schwann cell characteristics, capable of eliciting regeneration and re-myelination of PNS and CNS nerve fibers [17].

MSCs were treated with beta-mercaptoethanol (BME) followed by retinoic acid (RA) and cultured in the presence of FSK, bFGF, platelet-derived growth factor (PDGF) and neuregulin [17] (Fig. 1). Such differentiated cells were morphologically different from the original untreated MSCs but were quite similar to Schwann cell morphology, and expressed p75, GFAP, S-100, O4 and P0, known markers of Schwann cells [17]. Immunocytochemistry showed that a higher ratio (approximately 90-95%) of induced cells were positive for S-100 and p75. Omission either of BME, RA, FSK or neuregulin from the induction procedure resulted in

incomplete differentiation both in morphology and immunoreactivity, showing that pre-treatment with the reducing agent BME and subsequent RA and trophic factor treatment are critical and required for MSC-derived cells to acquire competence for Schwann cell characteristics. The following sections describe the effectiveness of these induced Schwann cells in PNS and CNS injury models.

2.2. PNS Regeneration by M-Sch Transplantation

After a long distant gap was made in the sciatic nerve in adult rats, MSC-derived Schwann (M-Sch) cells were transplanted into the transected gap and PNS reconstruction evaluated by motor function, electrophysiology and histological analysis up to 6 months after transplantation [43].

M-Schs, labeled with a GFP expressing retroviral vector [17], were suspended in Matrigel at a concentration of $1-2 \times 10^7$ cells/ml and were transferred into permeable tubes to make artificial grafts. Adult rat sciatic nerve was completely transected at the sciatic notch and a 12 mm length dissected out of the distal nerve segment. A 12 mm long artificial graft, as described above, was interposed into the gap between the proximal and distal segments, and was anastomosed at both ends. After the operation, rats received adequate exercise and stretching every 3 days to prevent muscle contracture. At 6 months after transplantation, immunohistochemistry showed that transplanted M-Schs labeled with retrovirus-GFP were positive to P0 and MAG, reconstructed nodes of Ranvier, and remyelinated regenerating nerve axons. The number of regenerated axons in the axial section of the middle portion of the graft was significantly larger in the M-Schs transplanted group than the control Matrigel, without M-Schs, transplanted group.

Consistent with the reconstruction of nodes of Ranvier and myelination by transplanted M-Schs, functional recovery was recognized by walking track analysis and electrophysiological evaluation. The mean sciatic nerve functional index (SFI; see Bain *et al.* [4]) in M-Sch transplanted group showed a statistical improvement compared to control group ($p=0.03$). In the electrophysiological study, the M wave was recorded after stimulation of the sciatic nerve. Mean motor nerve conduction velocity (MNCV) was significantly improved in M-Sch group compared to control group ($p=0.01$) [43]. Although bone marrow stromal cells have a basic ability to differentiate into several types of cells, tumor formation was not found at 6 months after the engraftment under the histological observation [43].

These results showed that PNS could successfully regenerate both morphologically and functionally by transplantation of M-Schs, suggesting a great potential of M-Schs to develop an alternative therapeutic approach for the difficult reconstruction of a long distant gap in the peripheral nerve. While the PNS is known to regenerate when provided with artificial graft composed of non-cellular substances such as type I collagen and alginate [29, 78], it would be desirable to supply a plentiful amount Schwann cells or cells with Schwann cell-characteristics for the more efficient reconstruction of PNS tissue and functional recovery since these Schwann cells will lead faster and more abundant regeneration and myelin formation.

2.3. Axonal Regeneration in Spinal Cord Injury by M-Sch

M-Schs are also effective in promoting axonal regeneration and functional recovery in completely transected adult rat spinal cord [32].

Laminectomies were performed under deep anesthesia at the T7 and T8 levels in Wistar rats (8 weeks), and T6 and T9 spinal processes were clamped to fix the spine. The spinal cord was completely transected with microsurgical scissors at the T7 and T8 levels, and the T7 spinal cord segment was removed. A 2 mm diameter permeable tube filled with a mixture of Matrigel containing M-Schs (2×10^6 cells/each tube) as described above was transplanted into the gap between the rostral and caudal spinal cord stumps. For controls, only Matrigel was placed in the permeable tube [32]. After transplantation, the T6 and T9 spinous processes were tied together tightly with a 4-0 nylon suture to prevent kyphosis and maintain contact between the graft and spinal cord stumps.

Grafts had integrated well into the host spinal cords in the M-Schs transplanted group. In contrast to control group, the number of neurofilament (Sigma, 1:400)-positive nerve fibers was significantly larger in M-Sch group after 6 weeks ($p < 0.01$). Because the T7 spinal cord segment was completely removed and replaced by the graft, all of the nerve fibers within the graft can be considered as regenerated axons. A large majority of these nerve fibers were revealed to be tyrosine-hydroxylase positive fibers, while some of CGRP- and serotonin-positive fibers were also observed [32].

Hind limb function recovered in M-Sch group from 4 weeks after transplantation, and a significant difference compared to the control group was recognized in the BBB score up to 6 weeks after transplantation ($p < 0.01$). The average BBB score in the M-Sch group 6 weeks after transplantation was 7.0 (minimum 5 to maximum 10), which indicates all three joints of hind limbs had extensive movement. The best recovery score in the M-Sch group was 10, which indicates occasional weight supporting plantar steps, but no forelimb-hind limb coordination. In contrast, the average recovery score in the control group was 3.6 (minimum 2 to maximum 5), showing only two joints of hind limbs had extensive movement. Re-transection of the grafts at their mid-point in M-Sch 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 [32]. Accordingly, these results exclude the possibility that transplanted cells enhanced the activity of a locomotor pattern generator in the spinal cord, but rather emphasize axonal regeneration induced by transplanted M-Schs contributed to functional recovery.

2.4. Optic Nerve Regeneration by M-Sch Transplantation

The optic nerve is part of the CNS and is a readily accessible tract that contains the axons arising from retinal ganglion cells (RGCs). Cell bodies and axons can be manipulated separately. Such a relative anatomical simplicity has made the optic nerve a valuable model for investigating

CNS regeneration. We transplanted M-Schs *via* injection into the cerebrospinal fluid to promote survival, axonal regeneration and functional recovery of damaged RGCs.

One optic nerve (adult Wistar rat) was crushed at a point 1.5 mm behind the optic disc and M-Schs (1.0×10^6 cells) transplanted into the cerebrospinal fluid *via* the 4th ventricle [45]. Untreated MSCs were transplanted as a control.

In M-Schs group, GFP-labeled transplanted cells were detected around the crush soon after the treatment, and GFP-positive myelin was detected around optic nerve axons. The number of axons was significantly higher in the M-Sch-group compared with the control group. Furthermore, the number of retrogradely labeled RGCs was significantly higher than the control groups, suggesting that transplantation of M-Schs was effective not only to axonal regeneration but also to survival of retinal ganglion cell bodies.

These results suggest the transplantation of M-Schs is efficient for the reconstruction of retinal and optic nerve disorder, and emphasize that the axonal regeneration contributes to functional recovery.

3. NEURODEGENERATIVE DISEASES AND CELL TRANSPLANTATION

MSCs are effective in neuronal regeneration from two points: (1) They produce trophic factors in host tissue after transplantation, which is beneficial to the tissue protection and restoration. (2) MSCs have a potential to differentiate into neuronal cells, which may also be a source of neural replacement.

Untreated MSCs have been used in the treatment of injured spinal cord [13, 31, 45] and brain [7, 12], resulting in functional recovery owing to trophic effects. Hofster *et al.* [31] first reported that direct transplantation of intact MSCs into the spinal cord after an acute and subacute contusion injury promoted some functional outcome with improvements of gait. In most cases when untreated MSCs were transplanted, engrafted cells remained within the host tissue, although there was little distinct evidence that transplanted MSCs differentiated into functional neurons. The reason why intact MSC transplantation could improve some functions has been explained mainly by the production of trophic substances beneficial for nervous tissue such as neurotrophins, stem cell factors and interleukins [20, 30].

Other than the trophic and neuroprotective effects, MSCs have far greater potential to be applied to neurodegenerative diseases, due to their ability to differentiate into neural cells. Eglitis and Mezey first reported the influx of green fluorescent protein (GFP)-positive male bone marrow cells into the female brain and their differentiation into microglia and astrocytes [24]. Differentiation of injected murine bone marrow cells into NeuN and beta3-tubulin positive cells was also confirmed in irradiated normal hosts [8]. In addition, mouse enriched bone-marrow derived cells contributed to Purkinje neurons in damaged mice [51]. While these studies suggested plasticity of MSCs based on expression of neural specific markers, clonality and functions of these trans-differentiated cells was not estimated and has been suspected to partly rely on cell fusion or spontaneous differentiation with extremely rare frequency [2, 65].

Several reports have shown neuronal induction from MSCs *in vitro* [57, 74]. Woodbury *et al.* demonstrated that MSCs from rat and human can differentiate into neuron-like cells *in vitro* [74]. Other similar studies have also suggested that rodent MSCs differentiate into cells with neuroectodermal characteristics [34, 57]. However, the induction ratio of neurons has not been strictly estimated and sometimes being accompanied with the induction of glial cells. Furthermore, functional recovery with transplantation of these induced neural cells has not been tested in animal models of neurodegenerative diseases.

3.1. Induction of Neuronal Cells from MSCs

Recently we established new methods to systematically induce neuronal cells from human and rat MSCs. Highly efficient and specific induction of post-mitotic functional neuronal cells, without glial differentiation, can be achieved by gene transfer of Notch intracellular domain followed by the administration of certain trophic factors [19]. Additional administration of glial-cell line derived neurotrophic factor (GDNF) to those cells can efficiently induce tyrosine hydroxylase (TH)-positive and dopamine-producing cells [19]. Functional recovery could be obtained when grafted into a rat model of Parkinson's disease and stroke, suggesting that these induced neurons may have properties useful in the treatment of neurodegenerative diseases. We next describe neuronal induction system from MSCs and the possibility of clinical application to several kinds of neurodegenerative diseases.

The Notch signaling pathway has been known to influence cell fate determination during development, from maintenance of a pool of uncommitted precursors to the terminal specification of cells [37]. The mouse Notch1 intracellular domain (NICD) cDNA was subcloned into a pCI-neo, a cytomegalovirus (CMV) promoter-containing mammalian expression vector, and transfected into MSCs by lipofection followed by G418 selection. After transfection with NICD, MSCs substantially up-regulated markers related to NSCs and/or neuronal progenitor cells (NPCs), such as glutamate transporter GLAST, 3-PDGH and nestin [60, 76] suggesting that MSCs may acquire some of the characters of NSCs/NPCs when NICD is introduced.

Next, cells were subcultured once (60-70% confluence) with administration of certain trophic factors (bFGF, FSK and ciliary neurotrophic factor (CNTF)) for five days, which resulted in a highly efficient and specific induction of cells with neuronal characteristics, showing that approximately 96% of cells were immunopositive to MAP-2ab (Fig. 1). Non-transfected as well as control vector-transfected MSCs could not be induced to be MAP2-ab positive by the trophic factor induction alone, indicating that NICD transfection is necessary for MSCs to acquire neurogenic potential.

MSC-derived neuronal cells (MSC-Ns) extended neurite-like processes with abundant varicosities, and expressed neuronal markers such as MAP-2ab, neurofilament-M and beta-tubulin isotype3, although nearly 2% of nestin-positive cells could also be recognized. MSC-Ns did not proliferate when subcultured after trypsin treatment. Brd-U incorporation performed 5 days after trophic factor administration showed

that few MAP-2ab positive cells incorporated BrdU. In addition, less than 1% of MAP-2ab positive cells were immunoreactive to an intrinsic proliferation associated marker, Ki67. These results suggest that the majority of MSC-Ns were post-mitotic.

MSC-Ns were evaluated physiologically by using the voltage clamp method. Seven days after trophic factor induction, an outwardly rectified K⁺ current was elicited by positive voltage steps in MSC-Ns which was dramatically higher than that in untreated MSCs. Concomitantly, resting membrane potential became lower. However, the voltage-gated fast sodium currents, which represent functional neuron characteristics, could not be observed up to 14 days after trophic factor induction, suggesting that although MSC-Ns exhibit neuron-like morphology and express several neuronal markers, they, are not fully mature neurons but rather are in a process of maturation. Therefore, neurotrophins such as BDNF and NGF which were administered to MSC-Ns to promote maturation, presumably resulted in the generation of the sodium currents and action potential in the small population of MSC-Ns. These results indicate that, just after trophic factor induction, MSC-Ns are neuronal cells in a premature state which can be induced to become functionally mature neurons with further administration of neurotrophins [19].

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. As to GFAP, the signal was under detection level both in Western blotting and RT-PCR. From these results, the existence of glial cells among the final MSC-Ns population seems to be extremely unlikely and that only neuronal cells could be selectively induced from human and rat MSCs in this induction system [19]. In the following sections, application of MSC-Ns to neurodegenerative models are discussed.

3.2. Application to Stroke

MSC-Ns were transplanted into the infarction area in a left middle cerebral artery occlusion (MCAO) rat model reported by Longa [36]. The MCAO procedure was somewhat modified in our study and circling to the right and adduction of the right forelimb when lifted up by the tail were used as signs of successful left MCAO. Behavioral tests for functional restoration and histological assessment of an ischaemically damaged brain were conducted (unpublished data). Seven days after reperfusion, MSC-Ns were transplanted into the non-necrotic brain parenchyma. MSC-Ns were stereotactically injected into the left cerebrum at three locations, and thus, the total number of transplanted cells was approximately 40,000. The control group received only PBS without cell transplantation.

MSC-Ns transplanted rats showed significant recovery, compared to controls in vestibulomotor function, sensorimotor function and cognitive function test ($p < 0.01$) after 28 days. Histologically, there was no statistical difference in the mean infarct volume between MSC-Ns-transplanted and control group ($p > 0.05$). However, GFP-labeled transplanted

cells migrated from the injection site into the ischemic boundary area, expressed neuronal markers of neurofilaments, MAP-2ab and beta3-tubulin, integrated into the hippocampus and resulted in extended neuritis. In contrast, only a small number of cells (approximately 1%) were positive for GFAP.

Our results showed that MSC-Ns are effective in the amelioration of the rat stroke model. The potential of other kinds of stem cells, such as NSC and umbilical cord blood cells, for the usage of stroke has been reported [10,66]. Toda *et al.* reported the engraftment of NSCs into damaged hippocampus by transient global ischemia resulted in the partial improvement of impaired spatial learning in a water maze test [66]. However, 1-3% of the grafted cells survived and 3-9% of them expressed NeuN. Chen *et al.* reported that intravenous administration of human umbilical cord blood cells (HUCBCs), which contain many stem and progenitor cells, reduced behavioral deficits after stroke in rats [10]. However, the percentage of HUCBCs that expressed NeuN, MAP-2 and GFAP was $\leq 2\%$, $\leq 3\%$, and $\leq 6\%$, respectively. In addition, approximately 1-2% of intravenously injected HUCBCs were detected in the brain [10]. These reports indicate that only small fraction of NSCs or HUCBCs populations are expected to integrate into host brain, and that neuronal differentiation efficiency seems to be very low. Our study showed that approximately 30-45% of MSC-Ns survived in the host brain at one month after the transplantation and nearly 80% of them expressed the neuronal markers. Thus, the specific induction of neuronal cells from MSCs has great potential in cell transplantation therapy for stroke.

3.3. Application to Parkinson's Disease

For Parkinson's disease, transplantation of dopaminergic neurons is believed to be effective [33]. However, cells committed to express certain transmitters account for lower ratios in MSC-Ns [19]. For instance, the percentage of tyrosine hydroxylase positive cells was approximately 4%. As GDNF is known to be involved in the generation and development of midbrain dopaminergic neurons [1], GDNF was administered to MSC-Ns to increase the proportion of cells immunopositive for TH and resulted in nearly 40% of MSC-Ns becoming TH-positive cells. These GDNF treated MSC-Ns were referred to as TH-MSC-Ns. Then, the dopamine release upon depolarization *in vitro* was measured by HPLC using a reverse-phase column and an electrochemical detector system, showing that TH-MSC-Ns released dopamine to the culture media in response to high K⁺ depolarizing stimuli. These results indicate that functional dopamine producing neuronal cells could effectively be induced from MSCs [19].

In order to explore the ability of induced dopaminergic neurons to survive and function in the host brain, both rat and human TH-MSC-Ns were separately transplanted into the striatum in a rat model of Parkinson's disease. Unilateral administration of 6-hydroxy dopamine (6-OHDA) into the left medial forebrain bundle selectively destroys dopaminergic neurons in the substantia nigra, leading to quantifiable rotational behavior and providing a useful and commonly used model of Parkinson's disease [75]. Apomorphine-induced rotational behavior (mean rotation index = the mean

rotation number in post-/pre-grafting) was examined every two weeks until 10 weeks following cell implantation. 1×10^5 cells were grafted into the ipsilateral striatum. The control group received no grafting after 6-OHDA administration, which showed a rotational bias away from the lesioned side which persisted, whereas rats grafted with TH-MSC-Ns demonstrated substantial recovery from rotation behavior up to 10 weeks ($p < 0.01$). Mean rotation index was 1.3 ± 0.1 in control group, and 0.3 ± 0.1 in TH-MSC-Ns transplanted rats [19]. In addition, non-pharmacological behavior tests, adjusting step test [47] and paw-reaching test [44] were performed. Four and six weeks after grafting with TH-MSC-Ns, these rats showed significant improvement in both step adjustment and paw-reaching tests ($p < 0.01$). Immunohistochemically, grafted GFP-labeled TH-MSC-Ns were found to migrate and extend beyond the injected site and approximately 30% of cells remained in the striatum 10 weeks after transplantation. TH-positive processes extended to the outside of the implantation zone. The grafted striatum showed migration of GFP-positive transplanted cells that express marker of neurofilaments, TH and dopamine transporter (DAT). Among GFP-labeled cells, TH and DAT positive cells were approximately 45% and 30%, respectively. In contrast, most of GFP-labeled cells were negative to GFAP and O4, in consistent with *in vitro* data. Animals grafted were followed up to 16 weeks and there was no tumor formation observed in the brain.

Human TH-MSC-Ns were similarly transplanted into the striatum of 6-OHDA-lesioned rats. Animals were immunosuppressed with FK 506 daily, and rotational behavior was recorded at four weeks after cell transplantation. Grafting resulted in significant improvement in rotational behavior as well [19].

In summary, the additional administration of GDNF to MSC-Ns can efficiently induce TH positive, dopamine-producing cells. Striatal implantation of these rat- and human-derived cells reversed the rotatory behavior and improved stepping behavior and paw reaching tests in Parkinson model rats.

4. MUSCLE REPAIR AND CELL TRANSPLANTATION

Cell transplantation therapy also offers hope for the treatment of intractable muscle degenerative disorders. Although ES cells have been considered as candidates for transplantation therapy and may have great potential [3, 5, 21, 50, 55, 64, 68], they face limitations including problems of ethical concerns and tissue supply. Even though satellite cells, or muscle stem cells, can be isolated from adult and prenatal tissues [3, 21, 50, 68], the number of cells that can be harvested may be limited. Bone marrow is another source of myogenic stem cells [25, 64], although as the stem cell population is very small, the problem of inadequate tissue supply for therapeutic scale again arises.

4.1. Induction of Muscle-Lineage Cells from MSCs

We developed of a new method to systematically and efficiently induce skeletal muscle lineage cells with high purity from large population of MSCs, rather than a rare sub-population of myogenic stem cells contained in the bone marrow [18]. The induced population effectively different-

iated into mature myotubes with some cells persisting as satellite cells that continued to function in host muscle to restore degenerating muscles in the absence of repetitive transplantations [18].

Human and rat MSCs were firstly treated with cytokines of bFGF, FSK, PDGF and neuregulin for 3 days, and then transfected with a NICD expression plasmid by lipofection followed by G418 selection, and allowed to recover to 100% confluency (Fig. 1). At this stage, a large majority of MSCs developed to mononucleated myogenic cells expressing MyoD and myogenin. Cells were then supplied either with 2% horse serum or ITS (Insulin-Transferrin-Selenite)-serum free medium, both of which are known to promote differentiation of myoblasts to myotubes [77]. In addition, the supernatant of the original MSCs was also found to be an effective differentiation medium in the same efficiency. As horse serum is inappropriate for clinical usage, and cell survival and myotube formation were unsatisfactory in the ITS-serum free medium, MSC supernatant was used for the maturation medium. After administration of MSC supernatant, MSC-derived muscle lineage cells (M-MLCs) were obtained (Fig. 1). M-MLCs contained 3 kinds of muscle-lineage cells; (1) post-mitotic multinucleated myotubes: they expressed MyoD, myogenin and Myf6/MRF4 (a marker for mature skeletal muscle), skeletal myosin, myosin heavy chain, and troponin, exhibiting skeletal muscle characteristics. Some multinucleated cells exhibited spontaneous contraction *in vitro*. (2) mononucleated myoblasts: expressing MyoD and myogenin, and (3) satellite-like cells: immunopositive for Pax7 and c-MetR, both markers for muscle satellite cells [58]. To exclude non-muscle elements from M-MLCs, mononucleated myoblasts and satellite-like cells were subjected to clonal culture, and purified muscle-lineage cells could finally be obtained (clonal-M-MLCs).

4.2. Application to Muscle Degeneration Models

To estimate how workable these induced muscle lineage cells are in the repair of degenerated muscles, human clonal-M-MLCs were transplanted into immunosuppressed rats whose gastrocnemius muscles were damaged with cardiotoxin pretreatment [26]. Cells were labeled by means of a GFP-encoding retrovirus and then transplanted by local injection (L.I.) into muscles or by intravenous injection (I.V.). Two weeks after transplantation, GFP-labeled cells incorporated into newly formed immature myofibers, exhibited centrally located nuclei in both L.I. and I.V. 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. These findings indicate that clonal-M-MLCs are able to incorporate into damaged muscles and contribute to regenerating myofiber formation, regardless of the transplantation method [18].

Clonal M-MLCs contained satellite-like cells which developed into satellite cells in the host muscle. They were observed between the plasma membrane and laminin-positive basal lamina that surround distinct myofibers, and expressed Pax7 [58]. Muscle satellite cells are known to contribute to regenerating myofiber formation upon muscle

damage [6]. In fact, transplanted satellite-like cells were able to contribute to muscle regeneration as satellite cells *in vivo*. Four weeks after transplantation of human clonal-M-MLCs (I.V.), 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 clonal-M-MLCs to muscles of patients, those retained as satellite cells should be able to continue to contribute to future muscle regeneration [18].

Transplantation of muscle lineage cells is one of the potential therapeutic approaches for the treatment of muscle degenerative disorders such as Duchenne muscular dystrophy. Therefore, GFP-labeled human clonal-M-MLCs were transplanted into cardiotoxin-pretreated muscles of mdx-nude mice, which genetically lack dystrophin expression. Immunohistochemistry revealed the incorporation of transplanted cells into newly formed myofibers which expressed human dystrophin [18].

Compared to the various muscle stem cell systems that have been reported, the MSC system offers several important advantages. Since our induction system does not depend on a rare stem cell population, but can utilize the general population of adherent MSCs, which can be easily isolated and expanded, functional skeletal muscle cells can be obtained within a reasonable time course on a therapeutic scale. In 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, since they avoid the embryonic stem cell controversy.

At present, there are no effective therapeutic approaches for muscle dystrophy. Although the mechanism of muscle induction by NICD introduction remains to be clarified, we believe that our MSC differentiation system may contribute substantially to a major advance toward eventual cell-based therapies for muscle disease.

5. 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. Needless to say, bone marrow should at least be 'normal and healthy' for transplantation.

Even though transplantation of untreated MSCs are supportive to various kinds of degenerative models probably due to trophic supply, the frequency of spontaneous differentiation in the host tissue is extremely rare [2, 65]. Thus, from the point view of the cell based therapy, it is

desirable to develop a systematic induction system to obtain large amounts of purposive cells. Indeed, practical application to human degenerative diseases depends on the ability to control their differentiation into functional cells with high efficiency and purity.

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 [17-19]. 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 primates. In fact, recent reports raised the possibility of transformation in the long term cultivation of MSCs [56, 59]. Second, 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.

Fig. (2). Schematic diagram of “self-regenerative system” using MSCs. Neurons, Schwann cells and skeletal muscle cells induced from patients MSCs are transplanted back to their owner. Such self-regenerative system escapes not only from ethical problem but also from immuno-rejection.

Notch-Hes signaling are known to inhibit neuronal and myogenic differentiation in conventional development [37]. 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 [19]. 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 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 phenomenon.

Since MSCs can be obtained from patients, it is possible to establish a “self-regenerative system” (Fig. 2) using MSCs. 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 possible way to regulate MSC differentiation into functional Schwann cells, neurons and skeletal muscle cells which will be applicable to neurodegenerative, neuro-traumatic and muscle degenerative diseases.

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