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REVIEW ARTICLE

Mesenchymal cell populations: development of the induction systems for Schwann cells and neuronal cells and finding the unique stem cell population

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Abstract Mesenchymal cell populations, referred to as mesenchymal stem cells or multipotent stromal cells (MSCs), which include bone marrow stromal cells (BMSCs), umbilical cord stromal cells and adipose stromal cells (ASCs), participate in tissue repair when transplanted into damaged or degenerating tissues. The trophic support and immunomodulation provided by MSCs can protect against tissue damage, and the differentiation potential of these cells may help to replace lost cells. MSCs are easily accessible and can be expanded on a large scale. In addition, BMSCs and ASCs can be harvested from the patient himself. Thus, MSCs are considered promising candidates for cell therapy. In this review, I will discuss recently discovered high-efficiency induction systems for deriving Schwann cells and neurons from MSCs. Other features of MSCs that are important for tissue repair include the self-renewing property of stem cells and their potential for differentiation. Thus, I will also discuss the stemness of MSCs and describe the discovery of a certain stem cell type among adult MSCs that can self-renew and differentiate into cells of all three germ layers. Furthermore, I will explore the prospects of using this cell population for cell therapy.

Keywords Bone marrow stromal cells · Mesenchymal stem cells · Multilineage-differentiating stress-enduring cells · Stemness · Umbilical cord stromal cells

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Introduction

In 1968, Friedens et al. discovered the existence of non-hematopoietic cell populations in bone marrow, which are referred to as mesenchymal stem cells or multipotent stromal cells (MSCs). These cells are found in the mononucleated cell fraction of bone marrow, similar to hematopoietic stem cells (HSCs) and endothelial precursors (Thomas 2000), and they structurally and functionally support hematopoiesis. Although the expression of cell surface markers on MSCs varies with the culture conditions employed, the majority of this cell population express mesenchymal cell markers such as CD29 (β1 integrin), CD90 (Thy-1), CD54 (ICAM-1), CD44 (H-CAM), CD71 (transferrin receptor), CD105 (SH2), SH3, Stro-1, and CD13 (Pittenger et al. 1999, 2000). MSCs are easily accessible by bone marrow aspiration, can be isolated from the patients themselves, and are expandable on a large scale. For example, 1×10^7 MSCs can be obtained from 20–100 ml of bone marrow aspirate within several weeks, which is a sufficient amount for cell therapy. Compared with embryonic stem (ES) cells, there is no serious ethical problem for collecting MSCs, because there is no need to use fertilized eggs or fetuses. Thus, MSCs are considered a promising candidate for cell therapy.

Recently, MSCs have attracted increasing attention for the treatment of damaged tissues, for a number of reasons. One reason is that MSCs can protect the host tissue by providing trophic support and by regulating the immune response (Abdi et al. 2008; Kitada and Dezawa 2009). The second reason is the potential of MSCs to differentiate into other cell types to replace lost cells in damaged and degenerating tissues. Recent studies have demonstrated that mesenchymal tissues other than bone marrow, such as Wharton's jelly of the umbilical cord and adipose tissue,

possess cell populations with properties similar to those of MSCs derived from bone marrow (Hida et al. 2008; Kim et al. 2004; Musina et al. 2008; Prockop 1997; Romanov et al. 2003; Wang et al. 2004; Zuk et al. 2001). MSCs derived from bone marrow, umbilical cord, and adipose tissue are referred to as bone marrow stromal cells (BMSCs), umbilical cord stromal cells (UCSCs), and adipose stromal cells (ASCs), respectively.

Trophic effects of MSCs

The trophic effects of MSCs are exerted by a wide variety of trophic factors, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neuregulin-1, basic fibroblast growth factor (bFGF), insulin-like growth factor-1, and hepatocyte growth factor (HGF), which are secreted after grafting into damaged tissues, such as injured brain and spinal cord (Crigler et al. 2006; Mahmood et al. 2004; Yoshihara et al. 2007; Qu et al. 2007; Nakano et al. 2010). This trophic support provided by MSCs reduces apoptosis and enhances neovascularization, thereby contributing to tissue protection. Indeed, when transplanted into injured or degenerating tissue, in diseases such as stroke, spinal cord injury, and experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis, MSCs migrate to the lesion sites and contribute to histological and functional recovery (Yoshihara et al. 2007; Chopp and Li 2002; Chopp et al. 2000, 2008; Ohta et al. 2004a; Qu et al. 2007, 2008; Zhang et al. 2005, 2006; Ide et al. 2010). Consequently, BMSCs and ASCs have been applied in clinical trials for acute and chronic spinal cord injury in several countries. Our research group has reported that neural stem cells (NSCs) and BMSCs can be administered into the cerebrospinal fluid, through which these cells are able to migrate to the lesion sites, where they exert their trophic effects (Bai et al. 2003; Wu et al. 2002a, b; Ohta et al. 2004a, b). With this method, the transplanted BMSCs can elicit functional recovery, based on the Basso, Beattie, and Bresnahan score, which is the standard test to evaluate locomotor activity in the rat spinal cord injury model (Ohta et al. 2004a, Wu et al. 2003). This method has also been implemented in a clinical trial (Saito et al. 2008).

Immunomodulatory effects of MSCs

Because of the ability of MSCs to modulate the immune response, they are frequently considered *immune-privileged*. The immunomodulatory effect of MSCs was first recognized as their ability to suppress T-cell proliferation induced by mitogens for T cells, such as CD3/CD28, and alloantigens (Bartholomew et al. 2002; Le Blanc et al. 2003). MSCs can exert this suppressive effect on allogeneic and xenogeneic T cells. Soluble factors secreted by MSCs, including transforming growth factor β, HGF, prostaglandin E2, interleukin-10, and indolamine 2,3-dioxygenase, as well as inducible factors such as nitric oxide synthase and heme oxygenase-1, are thought to mediate this immunosuppressive effect (Tyndall et al. 2007; Meisel et al. 2004; Sato et al. 2007; Chabannes et al. 2007; Aggarwal and Pittenger 2005; Di Nicola et al. 2002). These factors also affect immune cell function, including cytokine secretion and the cytotoxicity of T cells and NK cells (Krampera et al. 2003; Rasmusson et al. 2003; Zappia et al. 2005; Spaggiari et al. 2006). These proteins also modulate the proliferation and maturation of B cells, and their ability to secrete antibodies (Schena et al. 2010; Asari et al. 2009; Tabera et al. 2008; Bochev et al. 2008; Corcione et al. 2006), as well as the maturation, antigen presentation ability, activation, and migration of dendritic cells (Ramasamy et al. 2007; Kronsteiner et al. 2011; Huang et al. 2010; Aldinucci et al. 2010; Magatti et al. 2009; English et al. 2008). In addition, MSCs are considered hypoimmunogenic because of their low expression of major histocompatibility complex (MHC) class I proteins and no expression of class II (Tse et al. 2003; Uccelli et al. 2006). These in vitro characteristics of MSCs are regarded as being beneficial for cell therapy. Indeed, many studies have demonstrated the survival of MSCs after transplantation, including xenotransplantation (Tse et al. 2003; Liechty et al. 2000; Niemeyer et al. 2006; Fibbe et al. 2007; Wei et al. 2009; Nakamura et al. 2007; Dai et al. 2005). Other studies, however, have observed the rejection of MSCs after allogeneic transplantation in mice (Nauta et al. 2006; Eliopoulos et al. 2005). Our study also showed that human BMSCs grafted into the injured peripheral nerve in rats were gradually rejected with mild immunosuppression (Shimizu et al. 2007). Differences in isolation methods, culture conditions, strains, transplantation methods, and animal models may have contributed to these conflicting findings (Abdi et al. 2008; Ryan et al. 2005). It should be noted that MSCs may not be immunologically privileged in all experimental paradigms or protocols, and thus the optimization of these methods is critical and indispensable for the application of MSCs for cell therapy.

Differentiation potential of MSCs

By the end of the twentieth century, it was known that BMSCs had the capacity to differentiate into cells belonging to the same hierarchical lineage as osteocytes, chondrocytes, and adipocytes (Pittenger et al. 1999; Prockop 1997). The similar differentiation potential of UCSCs and ASCs has also been demonstrated (Kim et al. 2004; Romanov et al. 2003; Zuk et al. 2001). The unusual plasticity of MSCs was first discovered in two in vivo studies (Mezey et al. 2000; Ferrari et al. 1998). Ferrari et al.

observed lacZ expression in skeletal muscle cells and satellite cells, the stem cell fraction of skeletal muscle, after transplantation of bone marrow cells derived from C57/MlacZ mice into the injured tibialis anterior muscle of scid/bg mice. After producing a secondary injury, nuclear lacZ expression was detected in skeletal muscle cells, as well as satellite cells, suggesting that the transplanted bone marrow cells differentiated into myogenic progenitor cells (Ferrari et al. 1998). Mezey et al. (2000) transplanted bone marrow cells derived from male mice into the intact brains of female mice and demonstrated the presence of the Y chromosome in neurons in the brains of female mice, suggesting that the transplanted bone marrow cells differentiated into neurons. The authors of these studies proposed that the MSCs spontaneously differentiated into skeletal muscle-lineage cells and neurons; however, the clonality of these cells was not demonstrated. Other studies have suggested that the spontaneous differentiation conjecture was due to incorrect data interpretation, and that the correct reason was the fusion of transplanted MSCs with host cells (Alvarez-Dolado et al. 2003; Terada et al. 2002). However, the cell fusion hypothesis cannot account in all cases for the frequency and ratio of MSCs integrated or their differentiation into cells with specific markers. Recent studies have demonstrated that MSCs can differentiate into endothelial cells and cardiac-lineage cells after transplantation through a mechanism independent of cell fusion (Harris et al. 2004; Kajstura et al. 2005). Spontaneous differentiation of MSCs after transplantation is still under debate at this moment, and a thorough evaluation is required to demonstrate spontaneous differentiation.

In contrast, studies performed in the last quarter of a century have elucidated the differentiation potential of MSCs with in vitro experiments; they have been shown to possess the potential to differentiate into skeletal muscle cells (Wakitani et al. 1995; Mizuno et al. 2002; Di Rocco et al. 2006; Gang et al. 2004), cardiac muscle cells (Makino et al. 1999; Qian et al. 2011; Yan et al. 2011; Kadivar et al. 2006; Rangappa et al. 2003), endothelial cells (Oswald et al. 2004; Cao et al. 2005; Gang et al. 2006), hepatocytes (Oyagi et al. 2006; Prindull and Zipori 2004; Snykers et al. 2009; Pournasr et al. 2011), neuronal cells (Mitchell et al. 2003; Sanchez-Ramos et al. 2000; Woodbury et al. 2000; Zuk et al. 2002; Safford et al. 2002; Kim et al. 2002), and insulin-producing cells (Tang et al. 2004; Timper et al. 2006; Chao et al. 2008), revealing the pluripotency of MSCs. While these findings clearly demonstrate the wide-ranging differentiation potential of MSCs, the ratio of cells differentiating into the target cell type is generally low. Thus, it may be difficult to obtain a sufficient number of cells for therapy. Recently, our research group has developed and improved on the novel efficient induction systems for generating peripheral glia, neurons, and skeletal muscle cells from BMSCs and UCSCs on a therapeutic scale (Dezawa et al. 2001, 2004, 2005; Hayase et al. 2009; Matsuse et al. 2010, 2011; Nagane et al. 2009; Shimizu et al. 2007; Wakao et al. 2010). In the following sections, the development of and recent improvements in induction systems for peripheral glial cells and neurons are summarized.

Induction of Schwann cells from MSCs and application to nerve repair

Schwann cells are the glial cells of the peripheral nervous system (PNS); they constitute the myelin sheath that envelopes the axon to achieve saltatory conduction of the action potential. Schwann cells possess great potential for nerve regeneration; the formation of bands of Bünger after the migration, proliferation, and activation of these cells in injured peripheral nerve and the production of basement membrane, both of which provide regenerating axons with the appropriate growth environment. In addition, Schwann cells secrete trophic factors, including NGF, BDNF, ciliary neurotrophic factor (CNTF), and bFGF, to rescue damaged neurons from apoptosis and facilitate the growth of regenerating axons. Owing to the physical and trophic support provided by Schwann cells, peripheral neurons can actively regenerate axons that can grow through the site of injury and participate in the functional recovery of the injured peripheral nerve (Dubovy 2004; Ide 1996; Chen et al. 2007; Geuna et al. 2010).

In contrast to PNS neurons, for a long time (ever since the observations of the pioneer neuroscientist Ramón y Cajal in 1928) it was believed that neurons in the central nervous system (CNS) do not to have the capacity to regenerate axons. However, Aguayo and his colleagues demonstrated that CNS axons in the rat brain and spinal cord could regenerate through peripheral nerve grafts that were up to 35 mm in length, thus demonstrating that CNS axons have the capacity to regenerate and that the inappropriate environment inhibits regeneration (David and Aguayo 1981; Benfey and Aguayo 1982). After these ground-breaking investigations, numerous studies have shown that Schwann cells can support the regeneration of axons and reconstruct the myelin sheath to contribute to the functional recovery of the damaged CNS (Kromer and Cornbrooks 1987; Harvey et al. 1995; Raisman 1997; Anderson et al. 1998). However, it was difficult to obtain a sufficient amount of Schwann cells for clinical application. In addition, another peripheral nerve must be sacrificed to harvest Schwann cells to treat the damaged nerve. Thus, there was an urgent need to establish a system in which cells with the capacity to behave like Schwann cells in the injured nerve could be efficiently induced from easily accessible sources and rapidly expanded.

Due to their ability to differentiate into numerous cell types, BMSCs were used for the development of the novel induction system for Schwann cells (Dezawa et al. 2001).

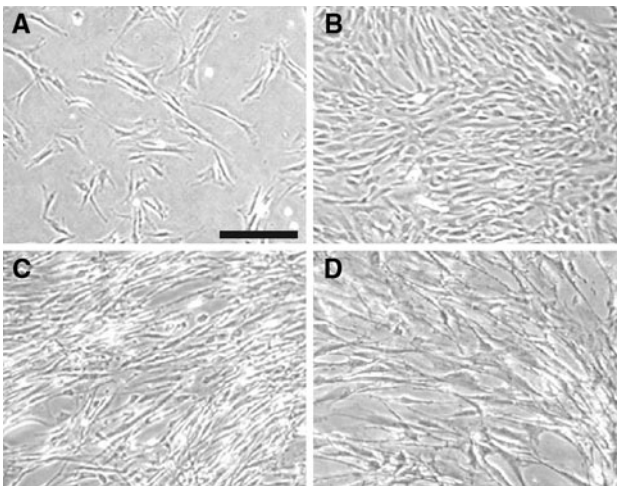

Fig. 1 Phase contrast images. Cell morphology of naive monkey BMSCs (**a**), monkey BMSC-derived Schwann cells (**b**), human BMSC-derived Schwann cells (**c**), and rat BMSC-Schwann cells (**d**). Morphological changes were evident from naive monkey BMSCs (**a**) to monkey BMSC-derived Schwann cells (**b**). Human and rat BMSC-derived Schwann cells exhibited similar morphologies to those of monkey. *Scale bar* 30 μm. This figure was reproduced from Wakao et al. (2010) with permission

This system can be applied to MSCs, including BMSCs derived from rat, monkey, and human, as well as human UCSCs (Dezawa et al. 2001; Matsuse et al. 2010; Shimizu et al. 2007; Wakao et al. 2010). MSCs were sequentially treated with β-mercaptoethanol and all-*trans*-retinoic acid, and cultured with soluble factors including forskolin (FSK), bFGF, platelet-derived growth factor, and neuregulin. Although BMSCs exhibit a spindle-shaped and elongated fibroblast-like morphology, they change their morphology to resemble native Schwann cells after induction (Fig. 1) (Wakao et al. 2010). Immunocytochemistry for Schwann cell markers, such as S100, P0, p75, glial fibrillary acidic protein (GFAP), L1, and O4, showed that while BMSCs did not initially express any of these markers, except for the slight expression of S100, all of these markers were expressed in the induced cells (Fig. 2) (Shimizu et al. 2007). Reverse-transcription polymerase chain reaction (RT-PCR) demonstrated that, initially, UCSCs did not express *P0* and *S100b*, slightly expressed *Sox10* and *Krox20*, and strongly expressed *Hath1*, the human homolog of *Atoh1*. During the subsequent treatment, *P0* and *S100b* began to be expressed, and *Sox10* and *Krox20* were up-regulated, while *Hath1* was down-regulated in the induced cells, suggesting that this induction system mimics the normal development of Schwann cells (Fig. 3) (Matsuse et al. 2010). The induced cells derived from the UCSCs were transplanted into the

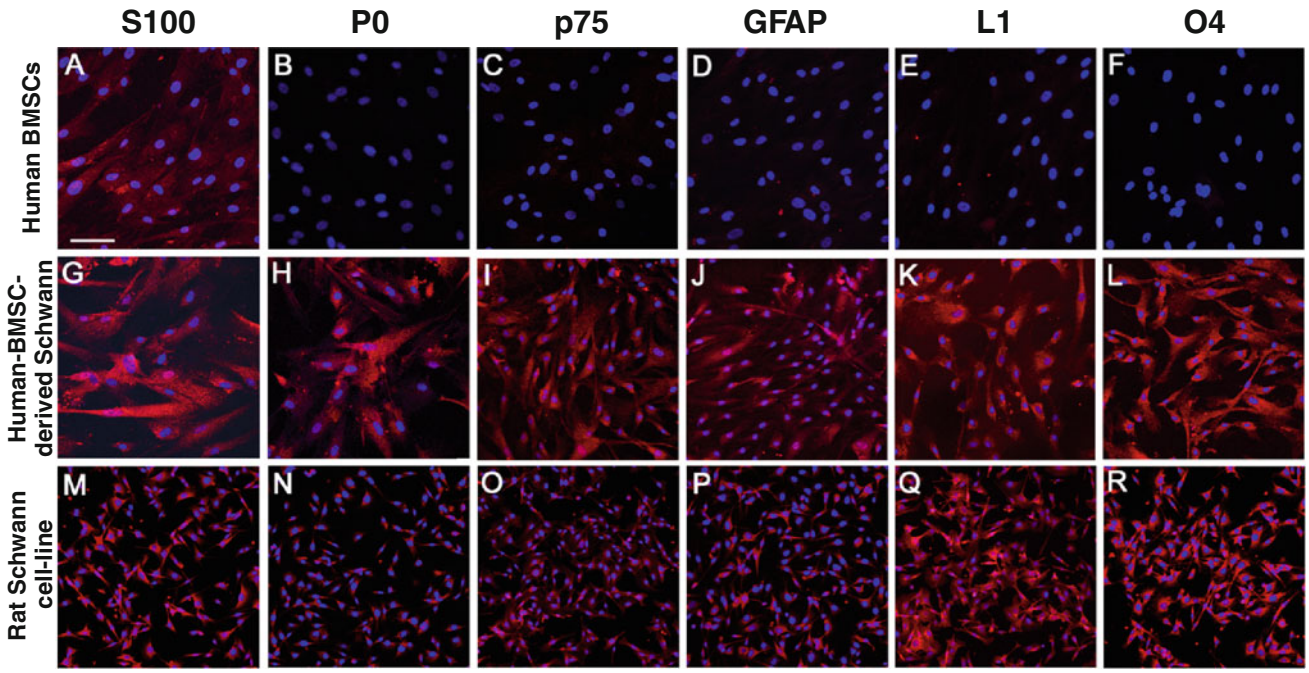

Fig. 2 Schwann cell marker expression. Immunocytochemistry for S100 (**a**, **g**, **m**), P0 (**b**, **h**, **n**), p75 (**c**, **i**, **o**), GFAP (**d**, **j**, **p**), L1 (**e**, **k**, **q**), O4 (**f**, **l**, **r**) in human BMSCs (**a**–**f**), human BMSC-derived Schwann cells (**g**–**l**), and a rat Schwann cell line (**m**–**r**). The untreated human BMSCs slightly expressed S100 (**a**) but were negative for other Schwann cell markers (**b**–**f**). After the induction, BMSC-derived Schwann cells became positive for P0 (**h**), p75 (**i**), GFAP (**j**), L1 (**k**), O4 antigen (**l**), and the immunoreactivity for S100 was up-regulated (**g**). The rat Schwann cell line was used as positive control for all these Schwann cell markers (**m**–**r**). Note that human cells (**a**–**l**) were much larger than rat cells (**m**–**r**). *Scale bar* 100 μm. This figure was reproduced from Shimizu et al. (2007) with permission

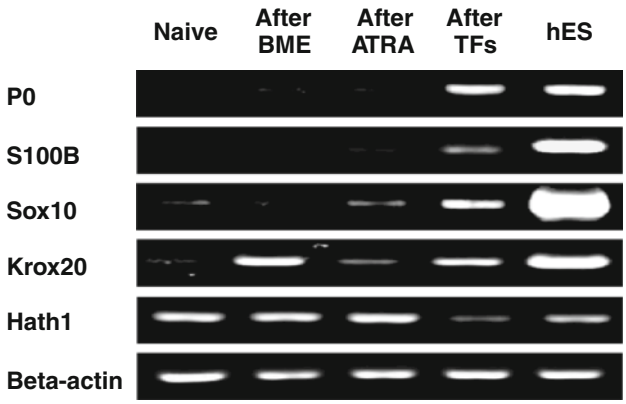

Fig. 3 RT-PCR analysis showing mRNA levels of molecules involved in Schwann cell differentiation in UCSCs and UCSC-derived Schwann cells. UCSCs were negative for P0 and S100B and were slightly or very faintly positive for Sox10 and Krox20 (*Naive*). After Schwann cell induction, mRNA expression of P0 and S100β (*S100B*) newly appeared in UCSC-derived Schwann cells (*After TFs*), and the mRNA levels of Sox10 and Krox20 were substantially increased (*After TFs*). Hath1, which was originally positive in UCSCs cells (*Naive*), was down-regulated along with the induction (*After TFs*). Human Schwann cells (hSCs) was used for the positive control. *ATRA* all-*trans*-retinoic acid, *BME* β-mercaptoethanol, *TFs* trophic factors. This figure was reproduced from Matsuse et al. (2010) with permission

severed sciatic nerve of rats, and recovery was estimated by histological and behavioral analyses. Gross anatomy of the graft showed that tissues inside the graft from both the UCSC-derived Schwann cell- and human Schwann cell-transplanted groups appeared whiter and looked more like neural tissue compared with tissue derived from the UCSC-transplanted group, although the tissue derived from the matrigel only-transplanted group displayed no parenchymatous tissue inside the graft (Fig. 4a–d) (Matsuse et al. 2010). Under low magnification, immunohistochemistry for the neurofilament protein, one of the components of the axon that constitutes the intermediate filament bundle, demonstrated that the extent of axonal regeneration in the UCSC-derived Schwann cell-transplanted group was significantly greater than that in the UCSC-transplanted group, and statistically similar to that observed in the human Schwann cell-transplanted group (Fig. 4e–g) (Matsuse et al. 2010). At a higher magnification, the green fluorescent protein (GFP)-positive transplanted cells localized inside the graft were in physical contact with the regenerating axons and expressed myelin markers such as myelin-associated glycoprotein, peripheral myelin protein 22, and periaxin 3 weeks after transplantation (Fig. 5) (Matsuse et al. 2010). Integration and myelination by the transplanted cells were also confirmed by immunoelectron microscopy for GFP (Matsuse et al. 2010). Behavioral testing, evaluated by walking track analysis based on the sciatic function index (Bain et al. 1989; Hare et al. 1992; Varejao et al. 2001), revealed greater functional recovery in the UCSC-derived Schwann cell-transplanted group than in the UCSC-transplanted group. No statistically significant difference was observed in the level of functional recovery between the UCSC-derived Schwann cell- and human Schwann cell-transplanted groups (Matsuse et al. 2010). Similar results were obtained by other studies, including xenotransplantation of human BMSC-derived cells into the severed sciatic nerve of rats, and autologous transplantation of monkey BMSC-derived cells into the severed median nerve (Shimizu et al. 2007; Wakao et al. 2010). In addition, long-term observations of monkey BMSC-derived Schwann cells autologously transplanted into the injured PNS revealed the safety and efficacy of this induction system, up to a period of 12 months. The safety of the grafted cells was evaluated by assessing general health, including weight monitoring and blood testing, such as blood cell counting and blood biochemistry; whole-body image analysis using ^{18}F-fluorodeoxyglucose-positron emission tomography; gross anatomy; and immunohistochemistry for cell proliferation markers. The efficacy was evaluated using electrophysiology, behavioral analysis, and immunohistochemistry against markers for axons and myelin (Wakao et al. 2010). Other research groups have confirmed that this Schwann cell induction system is also applicable to ASCs (Kingham et al. 2007; Jiang et al. 2008; Kaewkhaw et al. 2011; Faroni et al. 2011). Furthermore, the effects of Schwann cells, induced from BMSCs, on the promotion of axonal regeneration, myelination of regenerating axons, and functional recovery have been confirmed in the injured spinal cord (Kamada et al. 2005, 2010; Someya et al. 2008). These studies clearly show that Schwann cells can be induced from MSCs, including BMSCs, UCSCs, and ASCs, in vitro, and that these induced Schwann cells are functional—they are capable of enhancing axonal regeneration and myelination, and they contribute to the functional recovery of the injured nerve.

Induction of functional neurons from MSCs and their application to Parkinson's disease

Previous studies have shown that MSCs also possess the potential to differentiate into neuronal cells (Kim et al. 2002; Mitchell et al. 2003; Safford et al. 2002; Sanchez-Ramos et al. 2000; Woodbury et al. 2000; Zuk et al. 2002). However, the rates of differentiation of MSCs to neurons in these studies were not high. In addition, skepticism was raised against the particular method of neuronal induction (Lu et al. 2004; Neuhuber et al. 2004). Furthermore, previous protocols were inadequate to provide a sufficient amount of neurons for cell therapy.

We established a procedure in which neuronal cells could be specifically induced from MSCs (Dezawa et al. 2004). The mouse Notch-1 intracellular domain (NICD)

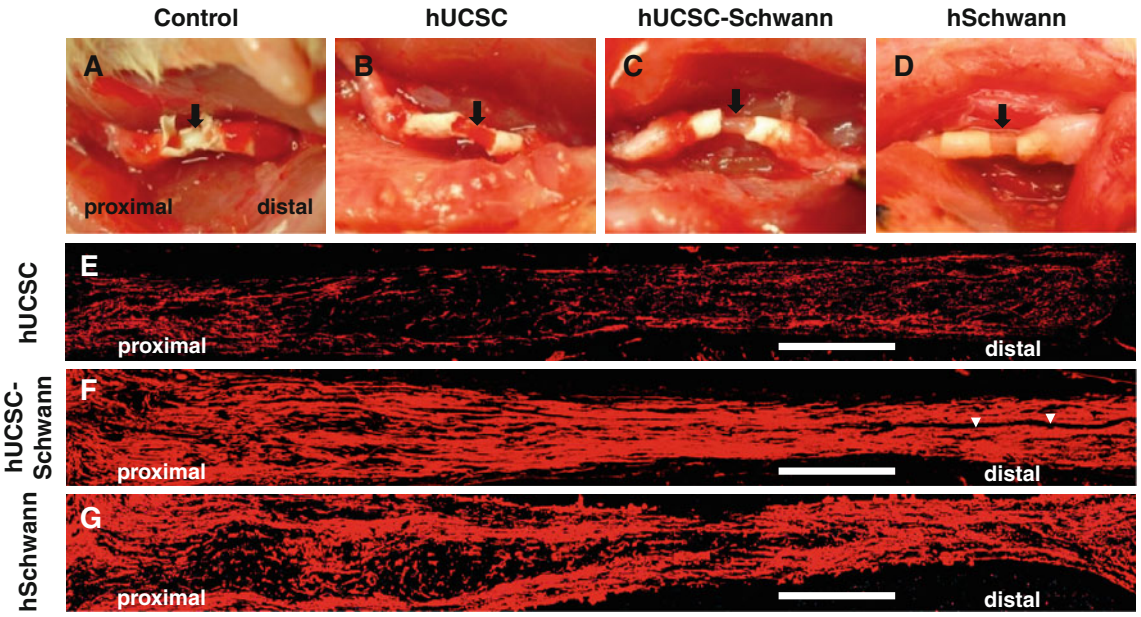

Fig. 4 Gross anatomy and immunohistochemistry against neurofilament in nerve grafts. **a–d** Macroscopic images of the grafts in the Matrigel-transplanted (*Control*), human UCSC-transplanted (*hUCSC*), human UCSC-derived Schwann cell-transplanted (*hUCSC-Schwann*), and human Schwann cell-transplanted (*hSchwann*) groups (**a–d**) after 21 days. In all animals, the anastomosis between the graft and sciatic nerve segment was tight and covered by augmented connective tissue. No parenchymatous tissue was found inside the graft in the control group (**a** *arrow*). Newly formed parenchymatous tissue was observed for the entire length of the graft in the UCSC, UCSC-Schwann, and hSchwann groups (**b–d**). The tissue inside the graft in the UCSC-Schwann and hSchwann groups (**c**, **d**) was whiter than that in the UCSC group (**b**). **e–g** Low-magnification images of the grafts immunostained for neurofilament. In the UCSC graft, there were small numbers of neurofilament-positive regenerated nerve fibers within the graft; a few neurofilament-positive fibers crossed the proximal segment, reaching the distal end of the graft (**e**). Neurofilament-positive fibers penetrated into the graft from the proximal side, and some of them reached the distal nerve segment in the UCSC-Schwann group (**f**) to an extent comparable to that observed in the hSchwann group (**g**) (*arrowheads*). *Scale bars* 1 mm. This figure was reproduced from Matsuse et al. (2010) with permission

was overexpressed in BMSCs by transfecting the *NICD* cDNA, subcloned into the pCI-neo plasmid, and *NICD*-transfected cells were selected with G418. The *NICD*-transfected BMSCs had a moderately reduced cell body size and thinner processes. Their gene expression profile also changed. The expression of the Notch-1 extracellular domain (*NECD*), the signal transducer, and activator of transcription 1 (*STAT1*), and *STAT3* was all down-regulated after *NICD* gene transfection (Fig. 6) (Dezawa et al. 2004). In the original protocol reported in 2004 (Dezawa et al. 2004), *NICD*-transfected BMSCs were treated with trophic factors such as bFGF, FSK, and CNTF in the adherent culture for several days. After treatment, these cells changed into compact, round-shaped cells bearing several thin processes, suggesting differentiation into post-mitotic neuronal cells (Fig. 7) (Kitada and Dezawa 2009). The differentiation of these cells into neurons was confirmed by immunohistochemistry for microtubule-associated protein 2 (MAP2), β-tubulin class III (Tuj-1), and neurofilament, showing a highly efficient rate of neuronal induction (approximately 96%). Electrophysiology with the patch clamp method revealed action potentials in these cells. The striking feature of this induction system is that the development of glial cells, including GFAP-positive astrocytes and O4-positive oligodendrocyte-lineage cells, is rare. The remainder of the cells were nestin-positive putative neural precursors that were not detected among untreated BMSCs. These findings strongly suggest that the BMSCs successfully differentiated into functional neurons. We then employed these induced neuronal cells in an animal model of neurodegenerative disease.

Parkinson's disease is a neurodegenerative disease characterized by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta, resulting in a reduction of dopamine in the corpus striatum. Rigidity, poor movement, tremors, and postural instability are the main symptoms of Parkinson's disease. Major therapeutic approaches are the administration of L-dopa and dopamine receptor agonists; and, in fact, these drugs are effective for some symptoms. However, the efficacy of these treatments gradually decreases over time, along with the loss of dopaminergic neurons (Curtis et al. 1984). Another method of treating Parkinson's disease is transplantation of human fetal dopaminergic neurons, which has been shown to be effective in alleviating neurological symptoms (Lindvall et al. 1994; Kordower et al. 1995; Freed et al. 1992).

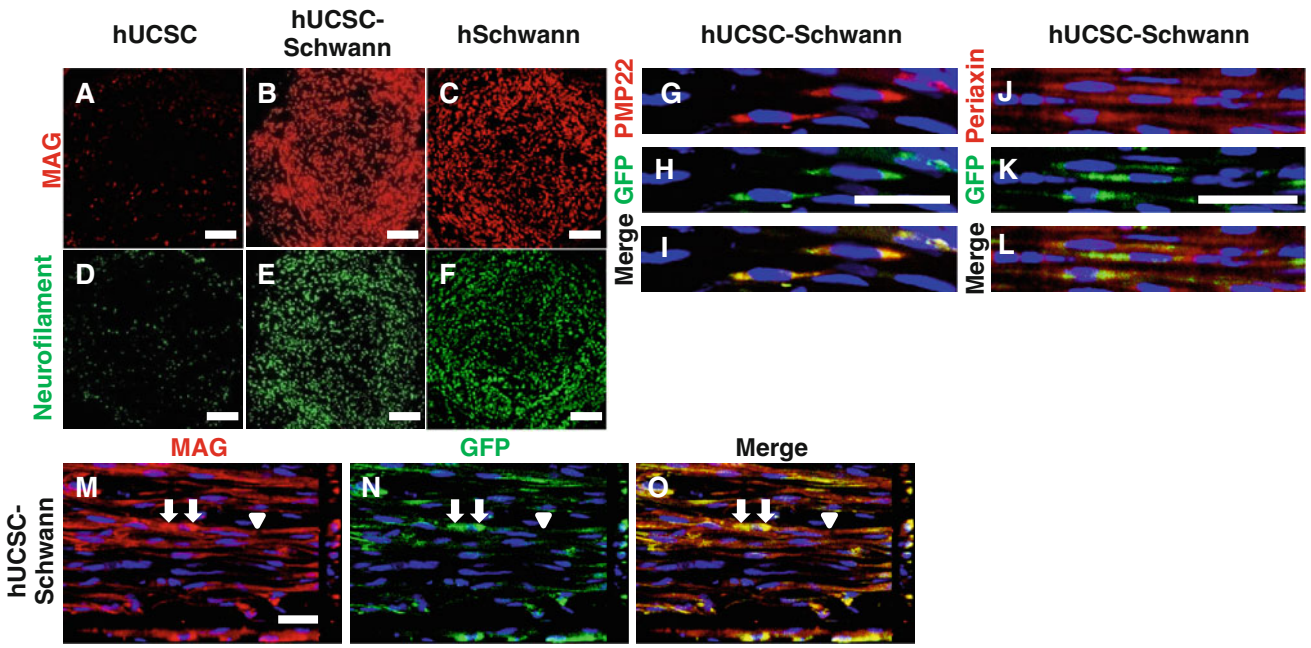

Fig. 5 Immunohistochemistry of the graft at 3 weeks after transplantation. **a–f** Images of transverse sections of the grafts in the human UCSC-transplanted (*hUCSC*) (**a**, **d**), human UCSC-derived Schwann cell-transplanted (*hUCSC-Schwann*) (**b**, **e**), and human Schwann cell-transplanted (*hSchwann*) (**c**, **f**) groups. Myelin-associated glycoprotein (*MAG*) (**a–c**) and neurofilament (**d–f**) are shown. In the graft of the hUCSC-Schwann group, there were more MAG-positive and neurofilament-positive fibers than those in the hUCSC group, to the same extent as observed in the hSchwann group. Images in **d–f** were taken via Alexa680 fluorescence and are shown as a *pseudocolor*. **g–o** Images of longitudinal sections of the graft in the hUCSC-Schwann group. GFP-positive transplanted cells also expressed peripheral myelin protein 22 (PMP22) (**g–i**) and periaxin (**j–l**). The three-dimensional constructed image showed that the GFP-positive transplanted cells in the UC-SC group (*arrows*) expressing MAG are involved in the formation of a node of Ranvier-like structure (*arrowheads*) (**m–o**). *Scale bars* **a–f** 100 μm, **g–o** 25 μm. This figure was reproduced from Matsuse et al. (2010) with permission

However, there are difficulties in using fetal cells because of ethical issues and limited supply. Thus, alternative approaches have been investigated for the treatment of Parkinson's disease.

MSCs can be an alternative source of dopaminergic neurons. Whereas our neuronal induction method using BMSCs was highly efficient at producing post-mitotic neuronal cells, the ratio of tyrosine hydroxylase (TH)-positive neurons, considered to be dopaminergic neurons, was just 3% after treatment with three different soluble factors (Dezawa et al. 2004). Glial cell line-derived neurotrophic factor (GDNF) is known to enhance the differentiation and survival of midbrain dopaminergic neurons (Lin et al. 1993; Akerud et al. 1999). Thus, we subsequently treated the cells with GDNF. This method was very effective, resulting in 40% of the cells being positive for TH (Fig. 7) (Dezawa et al. 2004; Kitada and Dezawa 2009). RT-PCR revealed that in the GDNF-treated cells, the *Nurr1*, *Lmx1b*, *En1*, and *Ptx3* genes, which encode the transcription factors that promote the differentiation of midbrain dopaminergic neurons, were newly expressed (Sakurada et al. 1999; Stromberg et al. 1993). High-performance liquid chromatography (HPLC) confirmed dopamine production and release into the culture medium in response to high-potassium depolarization. These findings clearly show that functional TH-positive dopamine-producing cells can be efficiently induced from BMSCs (Dezawa et al. 2004).

There are several models of Parkinson's disease. We chose to utilize unilateral intrastriatal injection of 6-hydroxydopamine (6-OHDA), an animal model for Parkinson's disease. In this model, the A9 dopaminergic neurons in the injected side of the substantia nigra pars compacta are selectively impaired, and abnormal rotational behavior emerges after subcutaneous injection of apomorphine. Thus, this method has been generally used as the animal model for Parkinson's disease. We transplanted TH-positive neuronal cells, induced from BMSCs, into the striatum of these animals and performed functional and histological analyses for a period of 10 weeks. Behavioral analysis, including apomorphine-induced rotational behavior, adjusting step test and paw-reaching test, demonstrated a significant improvement in animals receiving cell therapy. Histological analysis indicated that the GFP-labeled transplanted cells had integrated and differentiated into TH-positive neurons in the injected side of the striatum. HPLC analysis revealed the

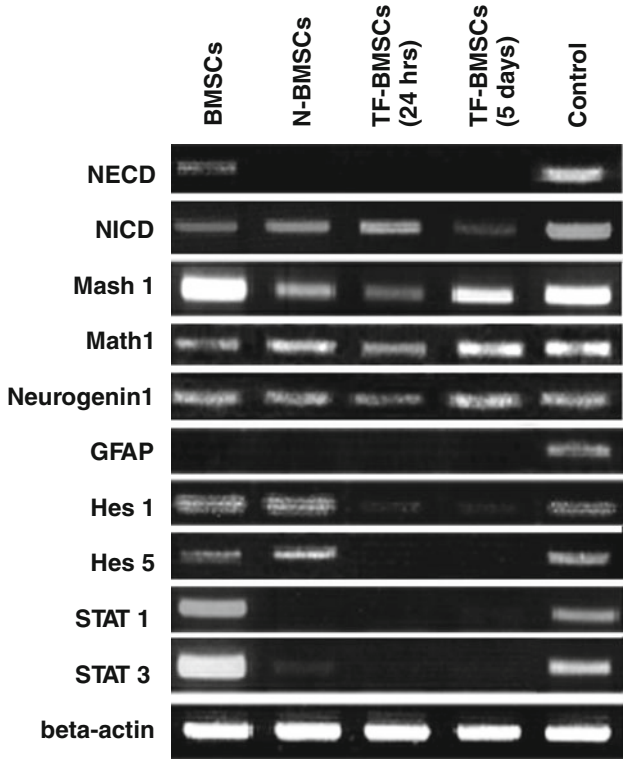

Fig. 6 The expression of transcription factors during the induction processes. RT-PCR of rat BMSCs, the NICD-transfected BMSCs (*N-BMSCs*), 24 h and 5 days after trophic factor induction in TF-BMSCs, and positive control (β-actin). This figure was reproduced from Dezawa et al. (2004) with permission

production and release of dopamine in slice culture. In addition, there were no signs of tumorigenesis in the animals analyzed up to 16 weeks. Transplantation of TH-positive neurons, induced from human BMSCs, into rats undergoing immunosuppressant therapy gave similar results. These in vivo findings clearly demonstrate the effectiveness, safety, and feasibility of using BMSC-derived dopaminergic neurons in the animal model of Parkinson's disease (Dezawa et al. 2004).

Improvement in the neuronal induction system

In the original protocol reported in 2004 (Dezawa et al. 2004), we introduced the *NICD* gene, in the pCI-neo plasmid, using lipofection. We tried to apply this method to BMSCs derived from the *Macaca fascicularis* monkey for testing the effectiveness and safety of this system for autologous transplantation. However, most of the transfected cells died because of the cytotoxicity of the lipofection reagents, indicating that modification of the protocol was required. In the previous study, we noticed that gene introduction using retrovirus or adenovirus did not result in neuronal cell induction from BMSCs. We interpreted this result as suggesting that the tentative and moderate expression of the transfected *NICD* gene was critical for the induction of neuronal cells (Dezawa et al. 2004). Thus, we developed the reverse transfection method, which achieved transient expression of the introduced gene (Nagane et al. 2009). The polyamine spermine was introduced into the natural water-soluble polysaccharide pullulan by an *N*,*N*′-carbonyldiimidazole activation method to yield spermine–pullulan. Polyion complex (PIC) was obtained by mixing the plasmid DNA with spermine–pullulan. PIC was dispersed onto succinic gelatin and ProNectin-coated culture dishes to adhere PIC. Reverse transfection was performed to apply BMSCs onto the PIC-coated culture dishes after removing PIC. Five key steps are needed for the expression of plasmid DNA: (1) attachment of the plasmid DNA onto the cellular surface; (2) internalization of the plasmid DNA into the cytoplasm; (3) endosomal escape of the plasmid DNA; and (4) transfer and internalization of the plasmid DNA into the nucleus. After 24 h, the Cy5-labeled pCI-*NICD* plasmid DNA was observed in the cytoplasm and nucleus of monkey BMSCs, suggesting that the five key steps listed above were achieved by the reverse transfection method (Fig. 8) (Nagane et al. 2009). Higher cell viability was observed with the reverse transfection method (97.0 $\pm$ 4.1%) compared with the lipofection method using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) (31.6 $\pm$ 1.4%). The luciferase assay demonstrated that there was no significant difference in the expression of the transfected gene between the two methods described above at 1, 3, and 5 days after starting the transfection. The optimum ratio of spermine–pullulan nitrogen atoms per plasmid DNA phosphorus atoms was 3:1 among the different ratios examined (1:1, 3:1, and 5:1). These findings demonstrate that the reverse transfection method provides higher cell viability than the lipofection method, with a similar efficiency.

We subsequently applied this reverse transfection method to our neuronal induction system. Three days after starting the transfection, transfected cells were selected using G418 for 5–7 days and treated with trophic factors including bFGF, FSK, and CNTF for 3 days. The induced cells exhibited round cell bodies with neurite-like thin processes, and they expressed the neuronal markers MAP2 and Tuj-1 (Fig. 9) (Nagane et al. 2009). With GDNF treatment for an additional 3 days, TH-positive neurons appeared. The production and release of dopamine by these TH-positive neurons were evaluated by HPLC under high-potassium depolarization stimuli (Fig. 9). We also applied this reverse transfection technique to BMSCs derived from other species, including mice and humans, and obtained similar results. These findings show that the spermine–pullulan-mediated reverse transfection technique is effective for the induction of functional dopaminergic neurons from BMSCs of various species, and that this technique will be applicable to the genetic manipulation of stem cells.

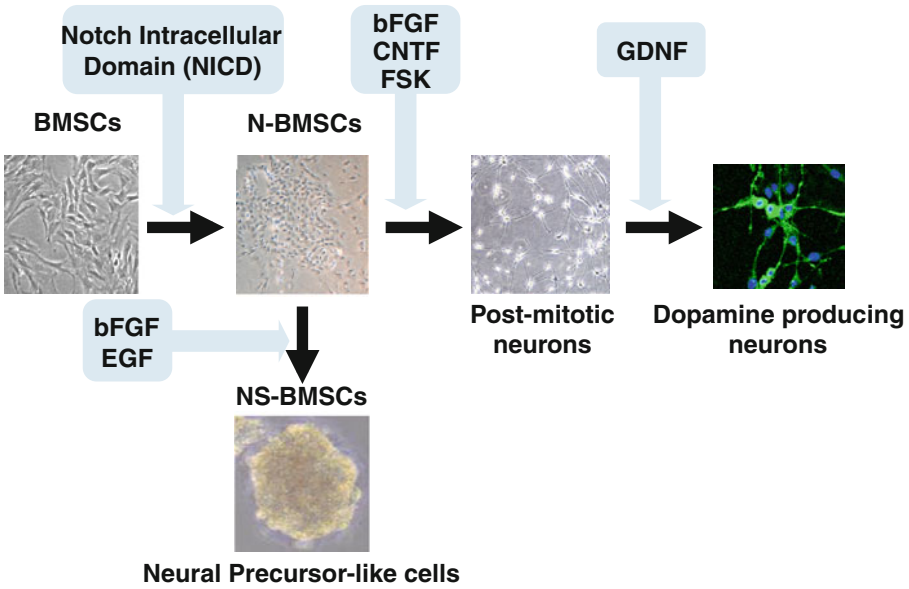

Fig. 7 Induction of neuronal cells from MSCs. After introducing the NICD gene, BMSCs change their properties, similar to neural precursor cells, but morphological changes in these cells are very small (*N-BMSCs*). As these cells were expanded at a lower cell density and treated with trophic factors including bFGF, CNTF, and FSK, N-BMSCs exhibited the properties of post-mitotic neurons, such as their action potential and neuronal marker expression after 5 days. GDNF treatment differentiates these cells into dopamine-producing neurons, which is applicable to a Parkinson's disease model. Alternatively, neurosphere-like spheres are induced from N-BMSCs in the suspension culture with bFGF and EGF (*NS-BMSCs*), which possess the neural precursor-like cells that can differentiate into functional neurons in vitro and in vivo. This figure was reproduced from Kitada and Dezawa (2009) with permission

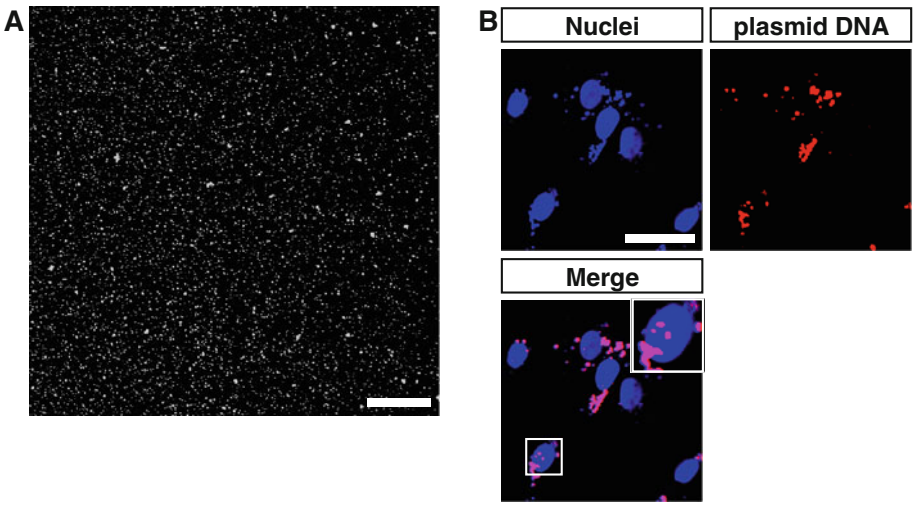

Fig. 8 **a** An image of the surface of a culture dish fixed with the polyion complex (PIC) containing Cy5-labeled plasmid DNA. Plasmid DNA was visualized using Cy5 fluorescence. **b** Confocal laser microscopic images of monkey BMSCs transfected with the Cy5-labeled plasmid DNA using the spermine–pullulan-mediated reverse transfection technique. The Cy5-labeled plasmid DNA could be found as punctuate signals in the cytoplasm and nucleus 24 h after starting the reverse transfection. Nuclei were counterstained using 4′,6-diamidino-2-phenylindole. *Scale bars* **a** 200 μm, **b** 100 μm. This figure was reproduced from Nagane et al. (2009) with permission

We are currently evaluating the efficiency and safety of this induction system for deriving neuronal cells from BMSCs, as well as the feasibility of autologous transplantation in the monkey model of Parkinson's disease.

The NSC culture method, which was established by Reynolds and Weiss (1992), enables these cells to be cultured while maintaining their stemness. With this protocol, NSCs form neurospheres in suspension culture in serum-

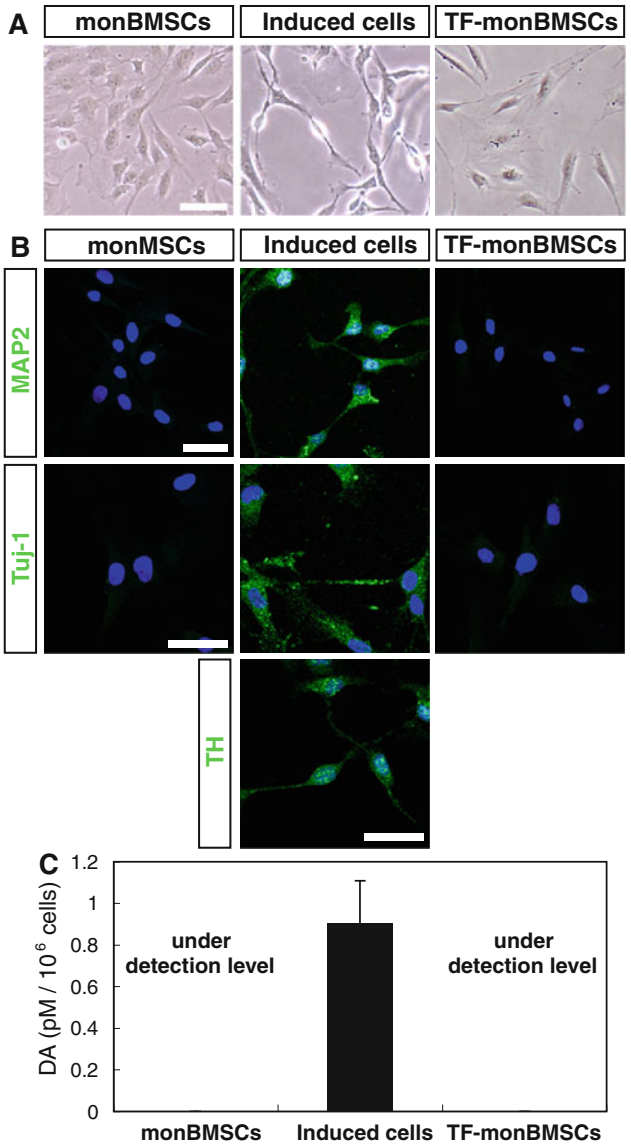

Fig. 9 Morphological and functional characterization of naive monkey BMSCs (*monBMSCs*), induced neuronal cells (*Induced cells*), and trophic factor-treated monBMSCs without the reverse transfection of pCI-NICD (*TF-monBMSCs*). TF-monBMSCs are shown as a negative control. **a** Phase contrast images. Induced neuronal cells exhibited round cell bodies with neurite-like processes. **b** Immunocytochemistry for neuronal markers. Induced neuronal cells were positive for the neuron-specific antigens MAP2 and Tuj-1. TH-positive neuronal cells detected after further treatment of induced neuronal cells with GDNF. Most of the naive monBMSCs and TF-monBMSCs were negative for MAP2 and Tuj-1. **c** Dopamine release assay detected using HPLC. Sequential treatment of low- and high-potassium conditions stimulated the secretion of dopamine. A substantial increase in dopamine secretion was detected only in induced neuronal cells (*middle column*), but dopamine secretion was under detection level in naive monBMSCs and TF-monBMSCs (*left and right columns*, respectively). Data were collected from three independent experiments. *Scale bars* 100 μm. This figure was reproduced from Nagane et al. (2009) with permission

free medium supplemented with defined factors. Trophic factors, including bFGF and epidermal growth factor (EGF), are also used in this culture method. In our original protocol for neuronal induction from MSCs, we utilized trophic factors such as bFGF, FSK, and CNTF after selecting *NICD* gene-containing MSCs in adherent culture. We altered this induction step to suspension cultures incubated with the trophic factors bFGF and EGF, similar to the culture method for NSCs, and found that neurosphere-like BMSC-derived spheres (NS-BMSCs) could be formed by this procedure 8 days after initiating suspension culture (Hayase et al. 2009; Kitada and Dezawa 2009). These spheres, formed by the *NICD* gene introduced-BMSCs, expressed markers for neural precursor cells, including nestin (61.0 ± 4.8%), NeuroD (96.8 ± 0.9%), and Sox2 (93.9 ± 2.4%), with much higher rates than naive BMSCs (2.3 ± 0.9, 9.4 ± 2.2, and 2.0 ± 1.4%, respectively) (Fig. 10) (Hayase et al. 2009). To evaluate the commitment of these cells to the neural lineage, differentiation of cells from these spheres was induced by a culture medium containing low serum and trophic factors, such as bFGF, FSK, and CNTF, similar to the original protocol for the adherent culture. Adherent rat and human BMSCs exhibited round cell bodies and thin neurite-like processes with abundant varicosities, and they expressed markers for post-mitotic neurons, such as Tuj-1 and MAP2 at high ratios (98.5 ± 0.5 and 95.7 ± 3.7%, respectively). In contrast, the ratio of GFAP-expressing astrocytes was very low (0.7 ± 0.3%). Cells positive for particular neuronal subtype markers, such as glutamate, TH, choline acetyl transferase (ChAT), serotonin, and gamma-aminobutyric acid (GABA), were also detected in the differentiated cells (Fig. 10). RT-PCR and real time-PCR confirmed the up-regulation of neuron-specific enolase as well as the expression of *ChAT* and voltage-gated sodium channel type III Scn3a in these cells. These results demonstrate the successful establishment of a highly efficient alternative method for obtaining neuronal precursors that can differentiate into functional neurons for the treatment of neural diseases. Next, we used these multipotent cells in a rat model of stroke (Hayase et al. 2009).

Recovery from stroke by grafting neurosphere-like spheres derived from BMSCs

Stroke is one of the major causes of death in adult humans, and is characterized by progressive neurological deficits. Effective treatments to restore lost neurological functions are currently unavailable (Bliss et al. 2007; Lindvall and Kokaia 2006). One of the potential strategies for treating

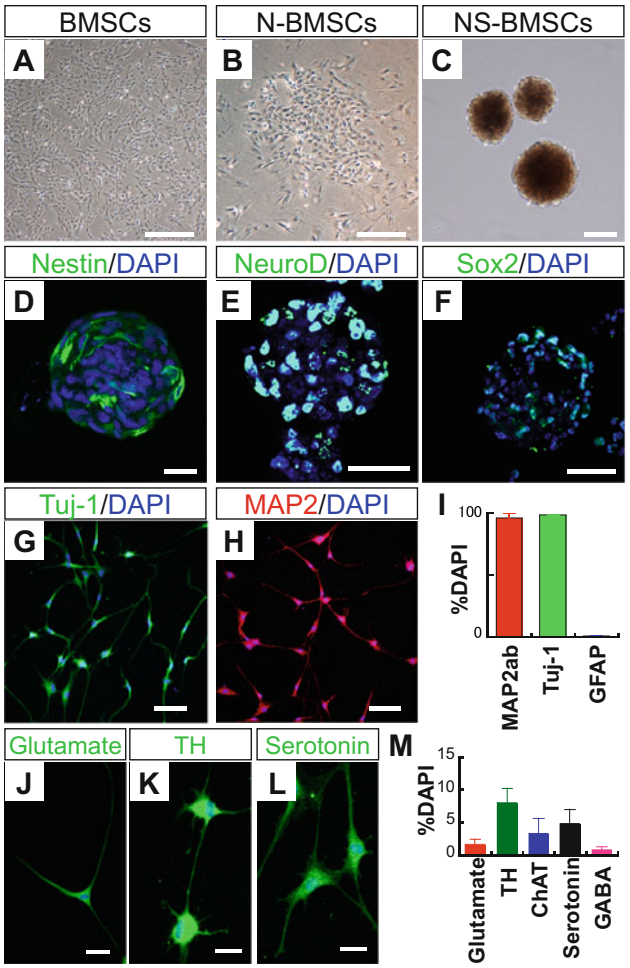

Fig. 10 Neuronal induction from BMSCs. **a–c** Phase-contrast images of rat cells. Original naive BMSCs (*BMSCs*) (**a**) changed their morphology after NICD transfection (*N-BMSCs*) (**b**). Spheres were made from N-BMSCs (referred to as *NS-BMSCs*) in the suspension culture. **d–f** Expression of nestin (**d**), NeuroD (**e**), and Sox2 (**f**) in rat NS-BMSCs. Tuj-1- (**g**) and MAP2- (**h**) positive neuron-like cells differentiated from rat NS-BMSCs. **i** The proportion of rat cells expressing neural markers. **j–l** Expression of glutamate (**j**), TH (**k**), and serotonin (**l**) in neuron-like cells derived from rat NS-BMSCs. **m** The proportion of cells expressing neurotransmitter-related markers in neuron-like cells derived from rat NS-BMSCs. *Scale bars* **a**, **b** 250 μm, **c** 100 μm, **d–f** 50 μm, **j–l** 20 μm. This figure was reproduced from Hayase et al. (2009) with permission

stroke is cell therapy with stem/progenitor cells that can replace lost cells. Transplantation of NSCs has been shown to be effective for the treatment of stroke (Chu et al. 2004). NSCs appear to restore lost neurological function through their ability to differentiate, secrete trophic factors, and stimulate endogenous cells. CNS cells, including neurons, astrocytes, and oligodendrocytes, can give rise to NSCs, which can replace lost host cells (Bithell and Williams 2005). Several types of trophic factors, such as BDNF and GDNF, are secreted by NSCs, which promote the survival of host neural cells (Bithell and Williams 2005; Iguchi et al. 2003). Exogenous NSCs can stimulate host NSCs to produce neural cells de novo (Bithell and Williams 2005; Wechsler and Kondziolka 2003). Thus, NSCs or other cells that possess NSC-like properties are potential candidates for treating stroke (Reubinoff et al. 2001).

As the spheres generated by our modified neuronal induction system resembled neurospheres, we transplanted NS-BMSCs into the infarcted area in the rat stroke model produced by middle cerebral artery occlusion (MCAo) (Hayase et al. 2009). The NS-BMSC-transplanted group displayed significant recovery in behavioral analyses for sensorimotor function (limb placing test) and cognitive function (Morris water maze test). Histological analysis revealed a broad distribution of GFP-labeled transplanted cells around the lesion and at the lesion boundary, but their migration was confined to the ipsilateral side. It was surprising that the number of GFP-positive transplanted cells (2.1×10^5 cells) was fourfold higher than the number initially transplanted (5.0×10^4 cells). Immunostaining for Ki67 revealed that 4.7% of the GFP-positive cells were proliferating at 14 days, and that no cells were positive for Ki67 100 days after grafting, suggesting that at least some of the cells were proliferating prior to transplantation, and that these cells stopped dividing 100 days following grafting. No sign of tumorigenesis was detectable up to 100 days after grafting. Immunohistochemistry for a neuronal marker, NeuN, and an astroglial marker, GFAP, demonstrated differentiation of the GFP-positive transplanted cells primarily into neurons ($79.5 \pm 0.1\%$) but occasionally astrocytes ($1.9 \pm 0.03\%$). Further histological analysis revealed that there were many GFP-positive neuronal cells expressing particular neuronal subtype markers; e.g., dopamine transporter, TH, glutamic acid decarboxylase, glutamate, calbindin, dopamine, adenosine 3′,5′-monophosphate-regulated phosphoprotein, and parvalbumin (Hayase et al. 2009). In addition, the GFP-positive transplanted cells exhibited immunoreactivity for synaptophysin around their cell bodies, suggesting synaptogenesis with the host neurons. These findings indicate that the transplanted NS-BMSCs differentiated mainly into neurons and expressed various neurotransmitter-related markers within the host brain to contribute to functional recovery up to 100 days after transplantation.

Compared with the fully differentiated post-mitotic neurons, NS-BMSCs have several advantages for transplantation. First, NS-BMSCs exhibit better survival, distribution, and integration within the host tissue. Previous studies showed that the survival of the transplanted cells was only 20–30% after transplantation, when fully differentiated post-mitotic neurons, derived from BMSCs, were transplanted (Dezawa et al. 2004; Mimura et al. 2005) into animal models of neurotraumatic and neurodegenerative diseases. When NS-BMSCs were transplanted, a fourfold

greater number of GFP-positive cells were detected. These findings suggest that the proliferative and migratory capacities of NS-BMSCs are attributable to their immaturity compared to fully differentiated post-mitotic neurons. Second, after transplantation, NS-BMSCs were positive for various transmitter-related markers within the host tissue, and were positive for synaptophysin around their cell bodies. These observations suggest that premature NS-BMSCs have greater flexibility to adapt to the host microenvironment; thus, they are able to differentiate into the appropriate neuronal types, according to the host microenvironment. Third, the preparation of NS-BMSCs is much easier than that of fully differentiated post-mitotic neurons from BMSCs, for which precise culture conditions, including the appropriate cell density at each culturing stage and the proper timing of trophic factor treatment, are critical. Therefore, the modified induction system for establishing NS-BMSCs is likely to be more feasible for treating acute diseases, such as stroke.

Recently we tried to combine this induction system for NS-BMSCs with tissue engineering techniques (Matsuse et al. 2011). Collagen sponges have been widely used as scaffolds for cell transplantation, and this material is already in clinical use (Teramachi et al. 1997). In addition, bFGF is known to promote neovascularization, which is necessary for cell survival, tissue restoration, and maturation of regenerating tissues (Masaki et al. 2002). Repeated infusion of a low dose of bFGF was reported to be more effective than a single high-dose injection (Seko et al. 2009). Thus, we transplanted NS-BMSCs in combination with a collagen sponge and gelatin microspheres incorporating bFGF that provide a sustained release of bFGF. Histological analysis performed 35 days after transplantation showed that the group which received NS-BMSCs with a collagen sponge and bFGF-containing gelatin microspheres (N + C + F group) exhibited significantly less infarct volume compared with the groups treated with PBS (PBS group), a collagen sponge only (C group), a collagen sponge with bFGF-containing gelatin microspheres (C + F group), NS-BMSCs only (N group), or NS-BMSCs with a collagen sponge (N + C group) (Fig. 11) (Matsuse et al. 2011). No massive space-occupying tissue was observed. However, the number of GFP-positive transplanted cells in the N + C + F group was about ninefold higher ($1.8 \pm 0.42 \times 10^5$ cells) than that originally transplanted (2.0×10^4 cells). This was significantly higher than in the N group ($3.4 \pm 2.1 \times 10^4$ cells), but not significantly higher than that in the N + C group ($1.5 \pm 0.39 \times 10^5$ cells). Immunohistochemistry for Ki67 suggested that most of the transplanted cells did not proliferate 35 days after transplantation. More than 95% of the GFP-positive transplanted cells in the N, N + C, and N + C + F groups exhibited immunoreactivity for neuron-specific markers, NeuN, and neurofilament. Significantly more von Willebrand factor-expressing cells were observed at the boundary of the injury cavity in the

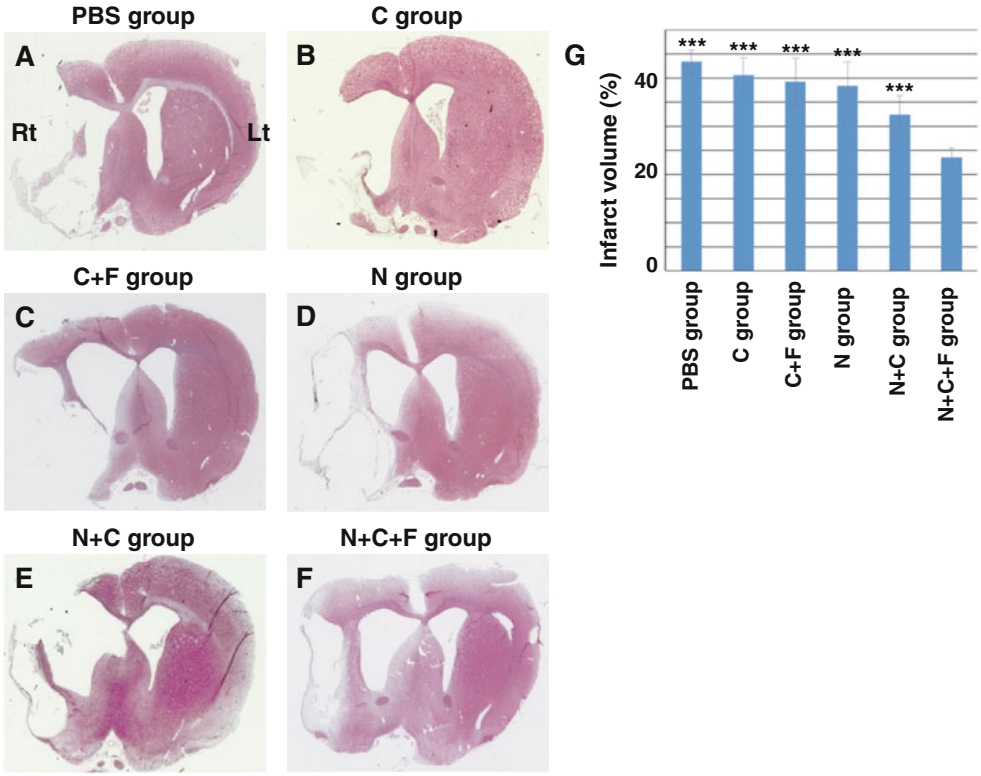

Fig. 11 The effect of BMSC-derived NS-BMSCs on the reduction in infarct volume. **a–g** Images of the coronal sections of the brain post-infarction from each group. **h** Summary of the mean lesion volume normalized to the volume of the contralateral hemisphere 35 days after transplantation. The N + C + F group had significantly smaller lesions than any other groups ($p < 0.001$). ***$p < 0.001$ compared to the N + C + F group. This figure was reproduced from Matsuse et al. (2011) with permission

N + C + F group than in the other groups, indicating enhanced neovascularization in the N + C + F group. The numbers of Pax6- and Sox2-positive cells around the ipsilateral side of the subventricular zone, where the host NSCs reside, were significantly greater in the N + C + F group than in the other groups, indicating stimulation of the proliferation of endogenous stem/precursor cells. Functional assessment throughout the experimental period demonstrated that there was significant recovery in the limb placement test (sensorimotor function) and the beam balance test (vestibulomotor function) in the N + C + F group compared with the other groups, and in the Morris water maze test (cognitive function) in the N + C + F group compared with four other (PBS, C, C + F, and N) groups. Based on the reduction in infarct volume, promotion of neovascularization, and stimulation of endogenous stem/precursor cell populations, it appears that NS-BMSCs and the two biomaterials exert a synergistic effect in this rat stroke model. These findings clearly demonstrate that NS-BMSCs are promising candidates for treating stroke when used in combination with a collagen sponge and bFGF-containing gelatin microspheres.

Why Notch?

As described above, we transfected the *NICD*-containing pCI-neo plasmid into BMSCs to induce differentiation into functional neurons. The Notch signaling pathway is known to be involved in cell fate determination during development, helping to maintain a pool of uncommitted precursor cell populations (Lundkvist and Lendahl 2001). In particular, Notch signaling appears to be an irreversible trigger for Schwann cell differentiation from neural crest stem cells (Morrison et al. 2000). Thus, we tried to stimulate Notch signaling in BMSCs by transfection, initially intending to obtain Schwann cells; but the result was, surprisingly, quite different from our expectation. Some BMSCs changed morphology, acquiring small round cell bodies bearing several thin processes. After one by one attempts, we established the novel induction system to derive neuronal cells from MSCs.

In contrast, recent studies have shown that Notch signaling inhibits the differentiation of BMSCs into neurons (Yanjie et al. 2007; Jing et al. 2011). In addition, Notch signaling has been shown to promote the self-renewal of and to inhibit the differentiation of NSCs into a more mature state. Notch signaling was also reported to promote the generation of astrocytes from neural precursor cells and to inhibit neurogenesis in collaboration with CNTF. In this process, the phosphorylation status of STAT3 at specific sites appeared to play a critical role (Nagao et al. 2007). We showed in our previous study that the expression of NECD, STAT1, and STAT3 was down-regulated following *NICD* gene introduction, although the expression of Hes1 and Hes5 was not affected (Fig. 6). Down-regulation of NECD likely indicates negative regulation of the endogenous Notch signaling pathway. Hes1 and Hes5 are known to exert the self-down-regulation effect. In addition, virus-mediated *NICD* gene transfer did not result in neuronal induction from BMSCs, suggesting that precise timing and the appropriate level of expression are needed for neuronal induction from BMSCs (Dezawa et al. 2004). Collectively, these data may be interpreted as follows: the precise timing and expression level of the *NICD* gene, mediated by exogenous lipofection-mediated delivery, inhibits endogenous Notch signaling, which, in turn, down-regulates Hes1 and Hes5 expression, which is further reduced by the self-down-regulation mediated by Hes1 and Hes5 themselves and trophic factor treatment. The down-regulation of Hes1 and Hes5 may lead to enhanced Mash1 expression, a neurogenic transcription factor. Recent studies have shown that a mesenchymal cell population, fibroblasts, can be converted into functional neurons by introducing certain transcription factors (Vierbuchen et al. 2010; Pang et al. 2011). Vierbuchen et al. (2010) reported that there was only one transcription factor, Mash1, which could promote neuronal differentiation from fibroblasts, although the rate of differentiation was higher when two additional factors were introduced. Thus, it appears that Mash1 plays a critical role in neuronal differentiation from mesenchymal cell populations, such as BMSCs and fibroblasts.

Stemness of MSCs and discovery of a novel stem cell population among MSCs

Although MSCs are frequently referred to as mesenchymal *stem cells*, their stemness has not yet been fully demonstrated. A stem cell should fulfill the following requirements: the potential for differentiation and the ability to self-renew. The potential for differentiation has been demonstrated by many studies, and has already been discussed in this review. Some studies showed the ability to self-renew through clonal analysis of MSCs, but the differentiation potential of these clonal cells was limited in mesodermal-lineage cells, such as osteocytes, chondrocytes, adipocytes, and muscle cells (De Bari et al. 2006; Sarugaser et al. 2009). As mentioned, MSCs behave like pluripotent cells because they can differentiate into cells of all three germ layers. Indeed, there are several reports proposing the existence of pluripotent cell subpopulations among MSCs, such as multipotent adult progenitor cells, marrow-isolated adult multilineage inducible cells, and very small embryonic-like cells (D'Ippolito et al. 2004; Kucia et al. 2006; Wojakowski et al. 2011). In all of these studies, the authors demonstrated the ability of these subpopulations to differentiate into cells of all three germ

layers; however, the ability to self-renew was not demonstrated for these cells. Thus, these pluripotent cells may not be able to meet the requirements of true stem cells. As MSCs are thought to consist of heterogeneous cell populations, the possibility that MSCs are a mixture of unipotent or multipotent stem/progenitor cells that can give rise to cells of one or a few germ layers cannot be ruled out. Another possibility is that MSCs contain a specific single cell population that can behave like pluripotent stem cells, in addition to the unipotent or multipotent cells described above. In both cases, MSCs, as a whole, can produce cells of all three germ layers. In the latter case, stemness, the ability for self-renewal, and the potential to differentiate into cells of all three germ layers, for the particular single cell population in question, should be demonstrated by clonal analysis. Recently, a specific single cell population with a potential similar to pluripotent stem cells was demonstrated by clonal analysis in adult human mesenchymal cell populations, including BMSCs and dermal fibroblasts (Kuroda et al. 2010).

Tissue stem cells can be induced to proliferate and differentiate so as to contribute to tissue repair under conditions of stress, burden, or damage. Thus, we conjectured that the purification or enrichment of certain stem cell types from mesenchymal cell populations could be achieved by exposure to stress conditions. Since stem cell fractions can be grown in suspension culture, where they form cell clusters in which their stemness is maintained (Kreso and O'Brien 2008; Reynolds and Weiss 1992), and we had already observed that, at a low frequency, MSCs spontaneously grew into cell clusters that were similar to cell clusters formed by ES cells, in which pigmented cells or hair-like structures are occasionally found (Fig. 12) (Kuroda et al. 2010), we cultured human MSCs—including human BMSCs and human fibroblasts—in suspension following treatment with six different stressors. Cell clusters with diameters of up to 50–150 μm were formed from MSCs after any one of these stressors, and they were positive for alkaline phosphatase staining. Cells that primarily localized to the outer surfaces of cell clusters whose diameters were >25 μm were found to be positive for pluripotency markers, such as Nanog, Oct3/4, Sox2, PAR-4, and SSEA-3 (Fig. 12) (Kuroda et al. 2010). Long-term trypsin (LTT) treatment gave the best results in terms of inducing the formation of the cell clusters described above. When human MSCs treated with LTT were cultured in single-cell suspension conditions after limiting dilution, about 10% of the cells formed single cell-derived cell clusters. Naive human MSCs could also form single cell-derived cell clusters, and the rate of cluster formation was about 1%. These cell clusters appeared to cease cell division 7–10 days after the start of suspension culture, but cell proliferation could be re-initiated by adherent culture conditions. When the adherent cells were dissociated and cultured as a suspension, single cell-derived cell clusters formed again, and the rate of cell cluster formation was about 50% when the adherent cells were allowed to proliferate up to 3000–5000 cells. Thus, cells with the capacity

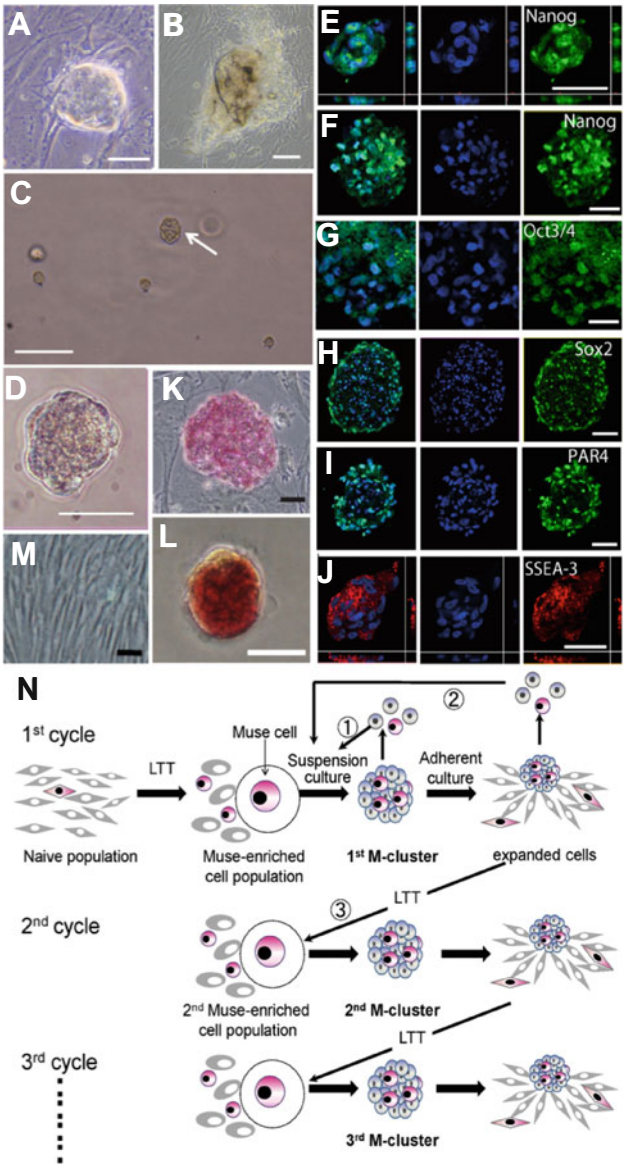

Fig. 12 Characterization of cell clusters. **a**, **b** Characteristic cell clusters that occur spontaneously in adherent cultures of naive human BMSCs. **c**, **d** The suspension culture of human dermal fibroblasts on day 7 showed cell cluster formation derived from a single cell (**c** *arrow*). **e**–**j** Immunocytochemical localization of Nanog (**e**, **f**), Oct3/4 (**g**), Sox2 (**h**), PAR4 (**i**), and SSEA-3 (**j**) in cell clusters formed by human fibroblasts (**e**, **i**, **j**) and human BMSCs (**f**, **g**, **h**). **k**–**m** Alkaline phosphatase reaction (ALP). Human ES cells (**k**) and cell clusters derived from human fibroblasts (**l**) were positive for and naive human fibroblasts (**m**) were negative for ALP staining. **n** Schematic diagram of the self-renewal of Muse cells. *Scale bars* **a**–**c** 100 μm, **d**–**m** 50 μm. This figure was reproduced from Kuroda et al. (2010) with permission

to form these types of clusters were expandable by repeating the following culture cycle: LTT, suspension culture, and adherent culture, resulting in the cyclical generation of cell clusters (Fig. 12). We confirmed that at least five generations could be successfully generated by repeating this cycle. These findings suggest that these cells maintain the ability to self-renew, in terms of the capacity to form cell clusters, under single-cell suspension culture conditions.

The cell clusters obtained from the single-cell suspension cultures were plated onto gelatin-coated dishes to assess their differentiation potential. These cells spontaneously differentiated into cells positive for neurofilament (an ectodermal-lineage cell marker), α-smooth muscle actin (a mesodermal marker), α-fetoprotein (an endodermal marker), cytokeratin 7 (an endodermal marker) or desmin (a mesodermal marker), as revealed by immunocytochemistry. RT-PCR also demonstrated the expression of α-fetoprotein, *GATA6* (an endodermal marker), *MAP-2* (an ectodermal marker), and *Nkx2.5* (a mesodermal marker). When transplanted into the testes of immunodeficient mice, these cells did not form teratomas, but integrated into the host tissue where the neurofilament-, α-fetoprotein-, or α-smooth muscle actin-expressing cells appeared. In addition, these cells were also labeled by an antibody specific for human mitochondria (Fig. 13) (Kuroda et al. 2010). This spontaneous cell differentiation was confirmed using cell clusters from both the first and third generations. These findings clearly demonstrate that cells with the ability to form cell clusters, and which express pluripotency markers, possess the ability to self-renew and the potential for differentiation into cells of all three germ layers. Because of their characteristics, these cells were named multilineage-differentiating stress-enduring (Muse) cells (Kuroda et al. 2010). Muse cells express stage-specific embryonic antigen (SSEA)-3 on their cell surfaces, which facilitates their isolation. Flow cytometry analysis demonstrated that Muse cells from human dermal fibroblast populations were distinct from other stem cell fractions residing in the dermis, such as skin-derived precursors, neural crest-derived stem cells, melanoblasts, perivascular cells, endothelial progenitors, and adipose-derived stem cells (Nishimura et al. 2002; Murga et al. 2004; Middleton et al. 2005; Crisan et al. 2008; Nagoshi et al. 2008; Fernandes et al. 2004; Biernaskie et al. 2009; Gimble et al. 2007), and under the appropriate culture conditions, the differentiation of these cells was shown to give rise to neural cells, hepatocytes, osteocytes, and adipocytes, with an efficiency greater than 85% (Wakao et al. 2011).

The findings described above suggested the possibility that Muse cells contributed to tissue repair as a consequence of their high stress tolerance and broad differentiation potential. Muse cells have been transplanted into pre-damaged tissue, including skin lesions, cardiotoxin-injected gastrocnemius muscle, and liver with CCl4-induced injury (Kuroda et al. 2010). The Muse cells homed in on the damaged sites, integrated into the host tissue, and differentiated into tissue-specific cells, such as human cytokeratin 14-positive epidermal cells in the epidermis, human dystrophin-positive mature skeletal muscle cells and Pax7-positive satellite cells in skeletal muscle, and human albumin- or human antitrypsin-positive hepatocytes

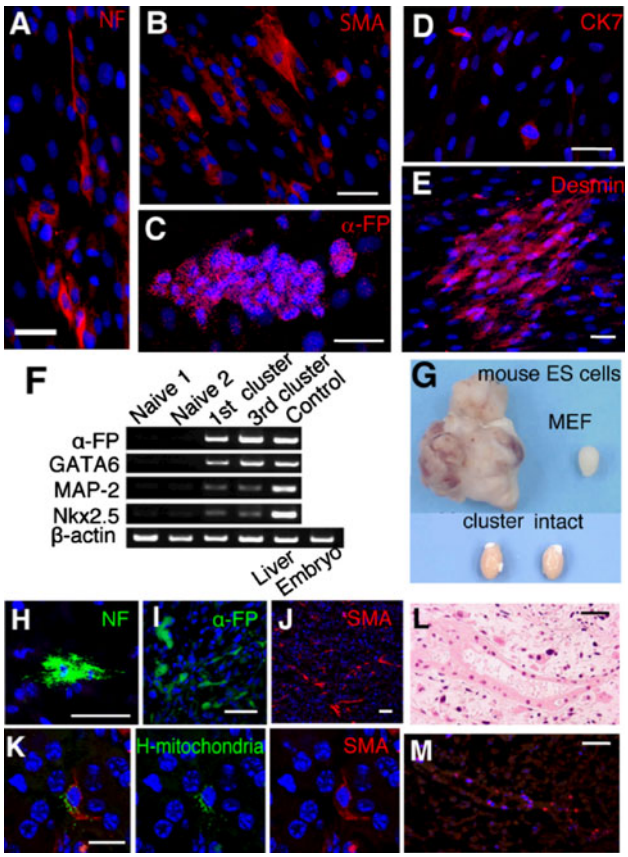

Fig. 13 Differentiation of Muse cells in vitro and in testes. **a–e** Immunocytochemistry against tissue-specific cell markers. Cultured cells derived from a single cell-derived cell cluster (human fibroblasts) were positive for neurofilament (*NF*) (**a**), α-smooth muscle actin (*SMA*) (**b**), α-fetoprotein (*α-FP*) (**c**), cytokeratin 7 (*CK7*) (**d**), and desmin (**e**). **f** RT-PCR analysis of naive cells and first- and third-generation cell clusters (*1st and 3rd clusters*) derived from human fibroblasts. Positive controls were human fetus liver (*Liver*) for α-FP and whole human embryo (*embryo*) for GATA6, MAP-2, and Nkx2.5. **g–m** Cell transplantation of cells derived from cell clusters into testes of immunodeficient mice. **g** Macroscopic aspects of uninjected testes (*intact*), testes injected with mouse ES cells (8 weeks), mouse embryonic fibroblast (*MEF*) cells (8 weeks), and cell clusters (6 months) (*cluster*). Immunohistochemistry for NF (**h**), α-FP (**i**), and SMA (**j**) in testes injected with cell clusters formed by LTT treatment. **k** Double-labeling of human mitochondria and SMA. Cells in the tube-like structure (**l**) were positive for human mitochondria in the adjacent section (**m**). *Scale bars* **a–e**, **h–l** 50 μm, **m** 20 μm. This figure was reproduced from Kuroda et al. (2010) with permission

in the liver, under the control of the microenvironment of each tissue. Thus, Muse cells are considered tissue-repairing stem cells that integrate into damaged tissue and differentiate into tissue-specific cells to contribute to tissue repair when transplanted into damaged/degenerating target tissues. In addition, Muse cells are not induced cells, but can be harvested directly from the tissue, in addition to the cultured mesenchymal cell populations. Thus, there is the possibility of developing a novel therapeutic strategy in which endogenous Muse cells are targeted specifically to the damaged tissue, and their differentiation into the appropriate cell types is regulated to optimize tissue reconstruction.

Induction of neurons from MSCs—differentiation or direct reprogramming?

Recent studies have reported that introducing a defined set of transcription factors leads to cell reprogramming that induces the formation of specific cell types, including neurons, cardiomyocytes, hepatocytes, and even pluripotent stem cells (Sekiya and Suzuki 2011; Efe et al. 2011; Vierbuchen et al. 2010; Takahashi and Yamanaka 2006). Because MSCs, such as BMSCs and dermal fibroblasts, are comprised of heterogeneous cell populations (Sorrell and Caplan 2004; Rider et al. 2007; Ratajczak et al. 2004), there might be several types of stem/progenitor cell populations among MSCs that have already committed to a specific cell lineage, such as skeletal muscle stem cells (Cornelison and Wold 1997), or have the potential to differentiate into cells of two or all three germ layers, such as neural crest-derived stem cells (Nagoshi et al. 2008) or Muse cells (Kuroda et al. 2010). These cells primarily give rise to the specific cell type by a cell differentiation mechanism, not by cellular reprogramming. In fact, when adult human fibroblasts are used, Muse cells are the primary source for the induction of induced pluripotent stem (iPS) cells (Wakao et al. 2011). In this study, Muse cells and non-Muse cells were isolated and transfected with defined factors, such as *Oct3/4*, *Sox2*, *Klf4*, and *c-Myc*, resulting in the exclusive induction of iPS cells. Although some colonies were formed from non-Muse cells, some of the major pluripotency markers were consistently not expressed in these colonies throughout the induction process. These colonies were also negative for *Abcg2*, *Dnmt3b*, and *Cdx2*, corresponding to the type I colony that is in an incompletely reprogrammed state and which cannot spontaneously transform into iPS cells (Chan et al. 2009). Gene expression profiles in Muse cells, Muse cell-derived iPS cells and non-Muse cell-derived colonies, compared with non-Muse cells, suggested that the introduction of this particular set of four factors influenced the expression of cell cycle-related genes, not pluripotency markers, whereas the expression of pluripotency markers by Muse cells was up-regulated after the induction of iPS cells. These data demonstrate that adult human fibroblast-derived, pre-existing Muse cells can transform into iPS cells after the introduction of a set of four specific factors (Wakao et al. 2011).

In contrast, Vierbuchen et al. (2010) introduced several transcription factors for the induction of functional neurons. They also reported that Tuj-1-positive cells, with a monopolar or bipolar morphology, occasionally emerged after the introduction of *Mash1*, and that no other factor by itself, other than Mash1, could induce these types of neuron-like cells. As mentioned, recent studies have revealed that the inhibition of the Notch signaling pathway can promote the expression of neuronal genes in BMSCs, in which Mash1 might be expressed, because the expression of the *NICD* gene was inhibited, indicating the down-regulation of *Hes1* and *Hes5*, the negative regulators of the *Mash1* gene (Yanjie et al. 2007; Jing et al. 2011). Yanjie et al. (2007) also demonstrated that 30% of the *Notch-1-shRNA*-transfected cells died by apoptosis. In addition, in our neuronal induction system, the vast majority of cells died during the selection of transfected cells by G418 treatment, in which the proportion of dying cells was much greater than that of untransfected cells (our unpublished data). In this case, there is the possibility that cells with the potential to receive appropriate signaling cues, which are likely stem or progenitor cells, are the only ones selected and capable of differentiating into neurons. The same process might take place during the formation of functional neurons, as was reported by Vierbuchen et al. (2010). If so, it is likely that the induction of neurons does not depend on reprogramming, but on cell differentiation, similar to iPS cell induction from adult human fibroblasts (Wakao et al. 2011). It needs to be clarified what factors determine the ability of a cell to respond to induction signals and its ability to transform into functional neurons, such as the cell's epigenetic state and differentiation potential, or any other unknown factors.

Conclusion

Because of their efficient expandability and versatility in repairing damaged tissue, MSCs are promising candidates for cell therapy. In addition, there are fewer ethical and safety concerns associated with MSC transplantation, similar to bone marrow transplantation. Indeed, MSCs have already been used in numerous clinical studies. However, most of these investigations focused on the trophic or immunomodulatory effects of MSCs, because the efficiency of differentiation was generally quite low. Our induction system is a practical solution for implementing

the ability of MSCs to differentiate into Schwann cells and neurons so that it can be applied in cell therapy for neurodegenerative and neurotraumatic diseases. As mentioned, specific stem cell populations have been identified among MSCs, which have a broad differentiation potential, the capacity for self-renewal, and the ability to efficiently repair damaged tissues. Further basic and preclinical studies will clarify the molecular and cell biology of MSCs in much greater detail, and will ultimately lead to the clinical application of a cell therapeutic approach that harnesses the remarkable differentiation potential of these cells.

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