REVIEW

Neural stem cells: progression of basic research and perspective for clinical application

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Abstract. It has long been thought that functional regeneration of the injured central nervous system (CNS) is impossible, as Santiago Ramóny Cajal described in the early 20th century, "once the development was ended, the fonts of growth and regeneration ... dried up irrevocably". In mammalian neural development, most neuronal production (neurogenesis) occurs in the embryonic stage. However, recent findings indicate that neurogenesis continues in the olfactory bulb, hippocampus, and dentate gyrus of adult mammalian animals, from the neural stem cells (NSCs). Recently developed techniques have made it possible to isolate, culture, and grow pluripotent self-renewing NSCs from both embryonic and adult brains. This basic research is attracting a lot of attention because of the hope that it will lead to regeneration and reconstruction therapy for the damaged CNS. In this review, recent findings on the stem cell biology of the CNS and strategies for its potential therapeutic application will be discussed. (Keio J Med 51 (3): 115–128, September 2002)

Key words: regeneration, prospective identification, adult neurogenesis, transplantation, embryonic stem cell

Neural stem cells (NSCs) are defined as undifferentiated cells that are able to self-renew as well as generate the three major cell types that constitute the central nervous system (CNS): neurons, astrocytes, and oligodendrocytes (pluripotency) (Fig. 1). Notably, the presence of NSCs or neural stem-like cells is not a recent concept, and a model close to the present one had already been proposed at the end of the 19th century: "there are neural precursor cells in the ventricular zone of the neural tube, which is the primordium of the CNS at the developmental stage (embryonic stage), and these precursor cells differentiate to neurons and glia".[1] These neural precursors located at the ventricular zone of the fetal neural tube were observed in more detail in the 1960's and named "matrix cells".[2] Building on the great achievements of our predecessors 100 years ago, continued research and modern technology have resulted in important advances, including the development of both i) a selective culture method for NSCs and ii) an *in situ* identification method for NSCs using selective markers. Making full use of these two developments, studies with great impact have recently been made, which report the discovery of NSCs at unexpected stages and places (for example, in the adult brain) and surprising properties of NSCs. In this review, the basic biological properties of NSCs and the relationship of adult neurogenesis with higher brain function and psychiatric diseases are discussed. Although related topics, such as the regulation of NSCs' self-renewal and differentiation, the regional specificity of NSCs, and the acquisition of specific cell fates are very important, these issues are not described here, but they are discussed in other reviews.[3,4]

What Is an NSC?

Biologists studying stem cells of the hematopoietic system, small intestine, and skin described the basic properties of stem cells (somatic stem cells, not totipotential embryonic stem cells). These cells are: (a) undifferentiated, in which no specific differentiation marker is expressed, (b) able to proliferate, (c) able to self-maintain their population, (d) able to differentiate and generate many descendant cells with various functions, and (e) able to repair injured tissue.[5] However, it remains to be resolved whether stem cells in the ner-

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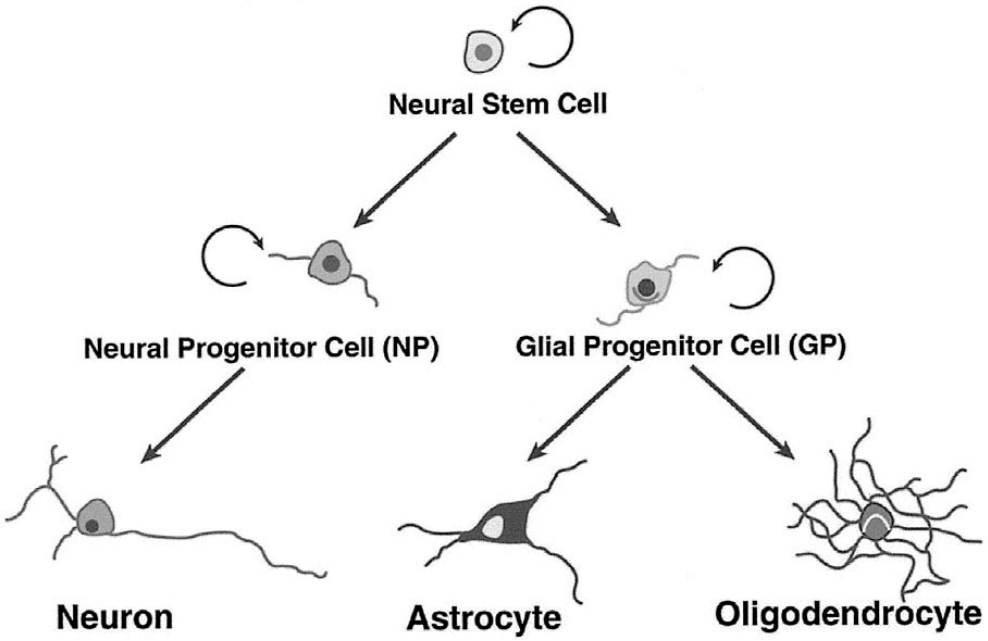

Fig. 1 Neural stem cell and its lineage. The pluripotency of a single NSC is often, but not always, mediated by the generation of cell-lineage-restricted intermediate progenitor cells, including neuronal progenitor cells (NP) and glial progenitor cells (GP); this has been shown both *in vivo*[6] and *in vitro*.[113] (Reproduced from Okano H: Seikagaku 2002; 74: 17–26 (in Japanese), Copyright © (2002), with permission from Nihon Seikagakukai).[114]

vous system strictly meet all these criteria; thus, the definition of NSCs differs slightly among individual researchers. In this review, I will consider the general characters of NSCs and progenitor cells to be as follows. The cell lineages generated from NSCs, which generate neurons, astrocytes, and oligodendrocytes differ among stages and regions of the CNS. In the cerebral cortex, NSCs generate intermediate progenitor cells that then generate specific cells[6]: neuronal progenitor cells generate only neurons and glial progenitor cells generate only glial cells, as shown in Fig. 1. In this cell fate process, pluripotential NSCs are committed to progenitor cells of a specific lineage. NSCs, neuronal progenitor cells, and glial progenitor cells are able to proliferate and are generally located in the periventricular area. When these three types of cells are not distinguishable, they are collectively called, ''neural progenitor cells''.

NSCs can be clonogenically expanded in free-floating ''neurosphere'' cultures. Researchers have used this system to define the NSCs experimentally and to quantify their multi-lineage potency and ability to self-renew (Fig. 2).[7] However, an NSC cannot be identified prospectively as a neurosphere-initiating cell. That is, when the culture is started, we cannot tell which cell will form a neurosphere. Only after the neurosphere is formed can we conclude that the initial cell was an NSC, *i.e.*, it is a retrospective identification.

Prospective Identification and Isolation of NSCs

Although there are highly selective marker molecules for NSCs, such as Musashi1 (an RNA-binding protein),[8–13] nestin (an intermediate filament)[14,15] and members of Sox-family (transcription factors),[16] these markers, unlike those for hematopoietic stem cells, are not cell surface antigens and therefore could not be used to identify viable NSCs prospectively using fluorescence-activated cell sorting (FACS), until recently.[3,17] To enable the prospective identification of viable NSCs, we first prepared a transgenic mouse (*nestin*-EGFP mouse) in which a fluorescent molecule, enhanced green fluorescent protein (EGFP), is expressed under the control of the 2nd intronic enhancer of the *nestin* gene, which acts highly selectively for NSCs. We then isolated populations of EGFP-expressing cells from the brains of these mice according to the expression level (*i.e.*, fluorescence intensity) using FACS.[18–20] The isolated cells were then investigated for the properties of NSCs, using mitotic activity (BrdU uptake), the efficiency of neurosphere formation, and self-renewal and pluripotency in low-density culture as indices. The GFP fluorescence intensity was correlated with these NSC-like biologic activities (Fig. 3).[18] Because this method concentrates NSCs using FACS, without involving *in vitro* growth-factor-induced expansion (*e.g.*, neurosphere culture), it can be used for

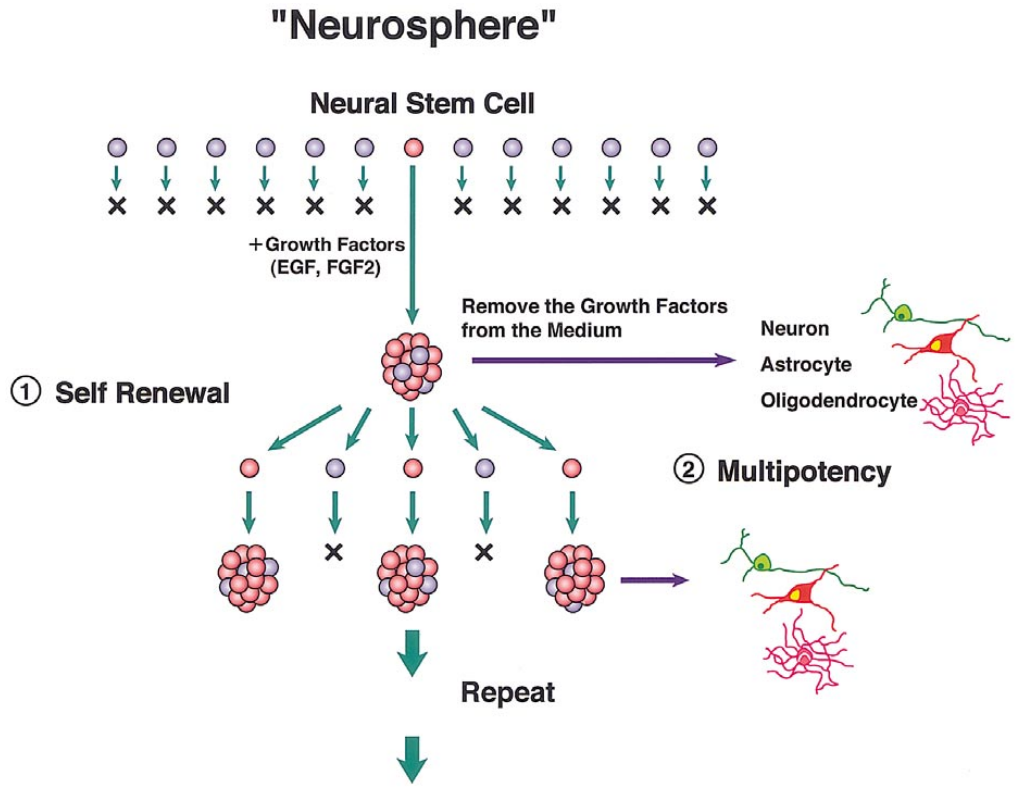

Fig. 2 Neurosphere formation assay. The major breakthrough for research on stem cell biology of the CNS was the development of the clonogenic expansion of NSCs in floating culture, called "neurosphere culture," within a serum-free defined medium containing EGF and/or FGF2.[7] A neurosphere derived from a single cell is capable of generating the major 3 lineages of the CNS, *i.e.*, neurons, astrocytes, and oligodendrocytes, indicating the multipotency of the neurosphere-initiating cell, upon the differentiation assay. If the neurosphere is dissociated into single cells, each cell starts to form a secondary neurosphere again with high frequency. (Reproduced from Okano H: Seikagaku 2002; 74: 17–26 (in Japanese), Copyright © (2002), with permission from Nihon Seikagakukai).[114]

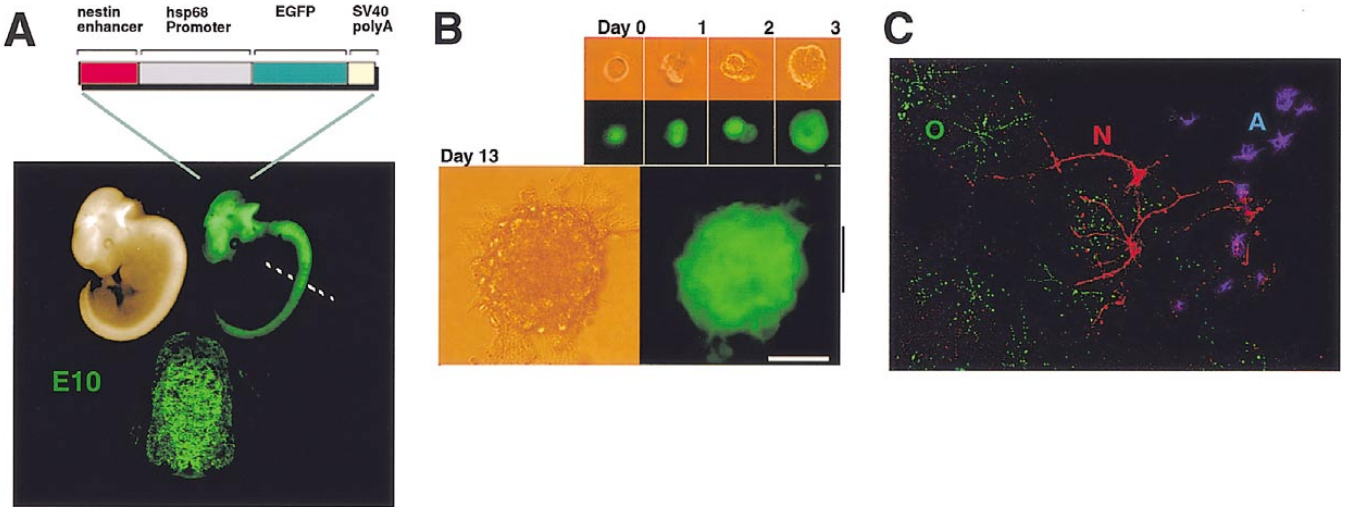

Fig. 3 *Nestin*-EGFP transgenic mice and isolation of NSCs. A) For the prospective identification and isolation of NSCs, a *nestin*-EGFP transgene was constructed by placing the EGFP cDNA under the control of the nestin 2nd intronic enhancer and minimum promoter of the hsp68 gene.[18] EGFP expression at embryonic day 10 of the *nestin*-EGFP mouse is shown below; it can be detected all through the neural tube without bias along the anteroposterior or dorsoventral axis. (right) Time lapse video recording of the neurosphere culture starting from a single *nestin*-EGFP cell. (Reproduced from Kawaguchi A, *et al*: Mol Cell Neurosci 2001; 17: 259–273, Copyright © (2001), with permission from Academic Press),[18] B) A single *nestin*-EGFP positive can give rise to neurons, astrocytes and oligodendrocytes upon clonal monolayer culture, indicating its multilineage potency.

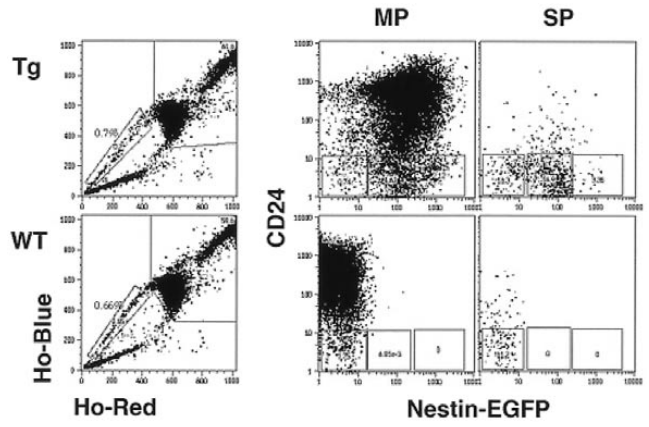

Fig. 4 *Nestin*-EGFP and CD24 expression in SP/MP cells. CD24-negative, *nestin*-EGFP positive, and SP cells are likely to be associated with NSC-activities, respectively.[18,25] Here, these three parameters are compared with one another, by characterizing the expression of CD24 (y-axis of the plots on the right) and *nestin*-EGFP (x-axis of the plots on the right) of the SP cells from mouse brain. The majority of mouse brain SP cells are *nestin*-EGFP positive and CD24 negative, although non-overlapping populations are also present. Thus, combinations of these 3 parameters might enable the further enriched isolation of NSCs compared with previous reports.[18,25] Alternatively, NSCs could exist in various populations in a heterogeneous fashion.[27] The original data was provided by Dr. Yumi Matsuzaki (Department of Physiology, School of Medicine, Keio University).

analyzing various biological properties of NSCs, such as their time of appearance and regional specificity, which have not been well characterized. Furthermore, because it allows the identification of viable NSCs, this method may be very useful for analyzing intrinsic (cell-autonomous) and external (non cell-autonomous) factors involved in the proliferation and differentiation of NSCs. It is also possible to introduce the same EGFP-transgene by lipofection or recombinant adenoviral vector into human brain-derived cells and to isolate neural progenitor cells using FACS,[21–23] and thereby supply donor human cells for therapeutic transplantation.

Within the past few years, there has been remarkable progress in determining surface antigen-based immunoselection strategies for sorting live NSCs.[24,25] Furthermore, stem-like cells can be purified from various organs as side population (SP) cells, based on their property to exclude Hoechst 33342.[26] To establish a more defined strategy for the prospective identification of NSCs, we have compared separation methods in detail cytometrically, characterizing the cell size (forward scatter; FSC)/granularity (side scatter; SSC) profile, and examining the *nestin*-EGFP expression, SP phenotype, and cell-surface antigens in developing mouse brains and found that the character of NSCs is not uniform and changes during development (Fig. 4).[27]

Localization of NSCs at Various Developmental Stages

Several studies have been performed to determine where and when NSCs exist within an organism. In this section, I describe the localization of NSCs in neurogenesis.

Before neurulation

When and where NSCs appear during ontogeny is important to understand the developing nervous system. In vertebrates, selective NSC markers, Musashi1[8,9,11,28] and *nestin*-EGFP,[18] are expressed over the entire neural plate before the neural tube closure, in both humans and mice. Fibroblast growth factor-2 (FGF-2)-responsive NSCs first appear *in vivo* at embryonic day (E) 8.5. Later, between E11 and E13, a separate and additive population of epidermal growth factor (EGF)-responsive NSCs arises from the earlier born FGF-2-responsive NSCs by asymmetric divisions.[29–32] However, it is not clear whether the first neural cells that appear during ontogeny upon ''neural induction'' emerge from the initial FGF-2-responsive NSCs (reviewed by van der Kooy and Weiss[33]).

After neurulation

It has been long believed that NSCs are located in the ventricular zone of the neural tube after neurulation.[2] This is very consistent with the results of immunohistochemical analysis using Musashi1 (Fig. 5). In the fetal neural tube, NSCs divide both symmetrically and asymmetrically.[34] In the former, one cell divides into two identical daughter cells. This division occurs at the stage when the number of cells constituting the neural tube rapidly expands (one neuroepithelial cell produces two neuroepithelial cells: proliferative symmetric division), before the generation of neurons begins. In asymmetric division, one cell divides into two different daughter cells. This division is necessary for generating the both differentiating cells and a stem cell by itself, and is a general characteristic of stem cells. In the mammalian CNS, NSCs (or neuronal progenitor cells that are able to generate only neurons) divide asymmetrically in the ventricular zone of the neural tube, and neural stem (or neuronal progenitor) cells themselves and neurons are generated simultaneously. After neuronal generation begins, neural stem (or neuronal progenitor) cells continue to divide asymmetrically and generate different types of neurons, depending on what micro-environmental and cell-autonomous factors are present.[35,36] The proliferative symmetric division of neuroepithelial cells is frequently observed at the relatively early stage of neural tube genesis in the forebrain region in mammalian development. In this type of cell

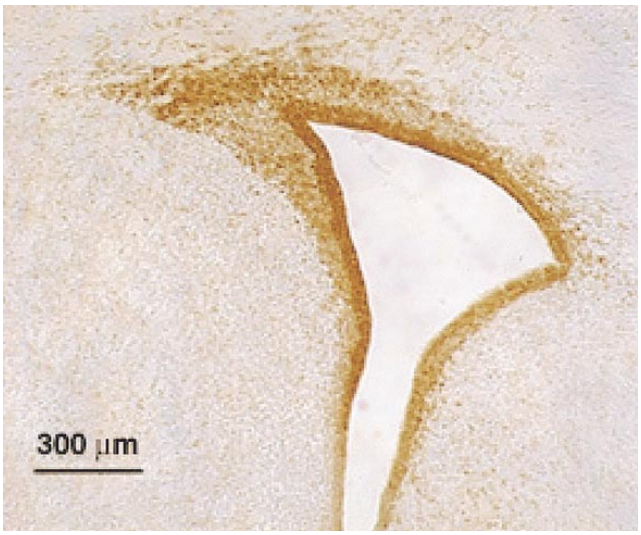

Fig. 5 Musashi1-expression in the periventricular region of neonate mouse brain. Musashi1-positive cells are present in the periventricular regions lining the lateral ventricle at postnatal day0 mouse forebrain. All through the development, Musashi1-expression can be observed in the periventricular zone.

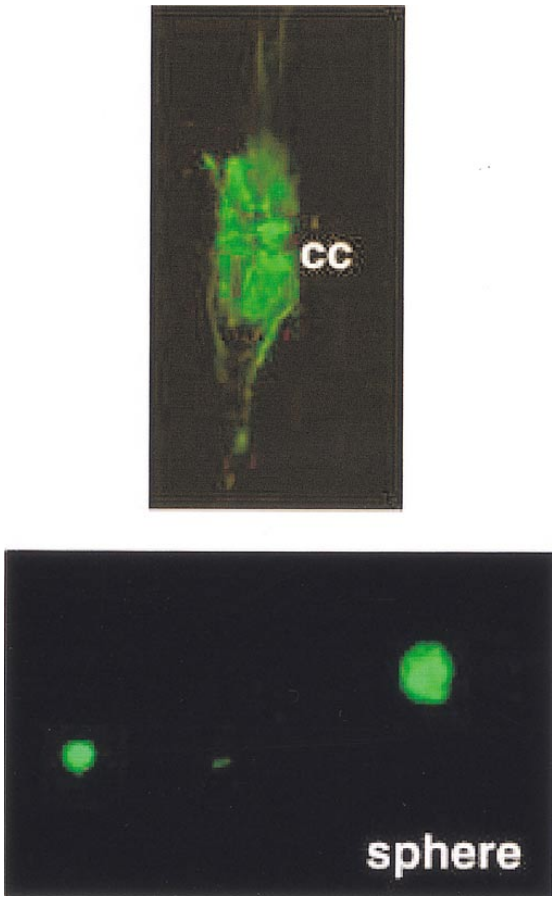

Fig. 6 *Nestin*-EGFP expression in the adult spinal cord. EGFP expression is seen in the ependymal cells surrounding the central canal, consistent with the previous reports indicating NSC activities in these cells.[48,52] These EGFP-positive cells are able to produce neurospheres *in vitro*.

division, the cleavage plane is vertical to the ventricular wall, whereas in asymmetrical division the cleavage plane is horizontal to the ventricular wall; however, the underlying control mechanism is still unknown.

In the embryonic tube, a portion of the NSCs (which can be called ''fetal NSCs''), have a characteristic radial morphology and are generally known as radial glia (reviewed by Barres).[37–40] Radial glia have long been thought to be involved in guiding or providing a scaffold for the migration of newly generated neurons.[41] Fetal NSCs are likely to play crucial roles in the histogenesis of the brain, in addition to the production of new neurons and glia (reviewed by Okano[4]).

Adult CNS

The generation of neurons does not just occur at the embryonic stage. It continues in adult rodents in two regions: the hippocampal dentate gyrus and olfactory bulb[42,43] (reviewed by Temple and Alvarez-Buylla, Gage, Alvarez-Buylla and Garcia-Verdugo[44–46]). The localization of NSCs at the neurogenic sites in adult brain has been shown by the selection culture method for NSCs, the neurosphere method, and *in situ* identification of NSCs using selective markers (Musashi1, nestin). It has been shown that interneurons in the olfactory bulb are generated from NSCs that are localized to the periventricular area facing the lateral ventricle and that neuronal cells move all the way to the olfactory bulb through a pathway called the rostral migratory stream[42,47,48] (reviewed by Temple and Alvarez-Buylla[45]). Furthermore, recent results indicate that, contrary to the current view, multipotential (neuronal-astroglial-oligodendroglial) precursors with stem cell features can be isolated not only from the SVZ but also from the entire rostral extension, including the distal portion within the olfactory bulb.[49] These findings demonstrate that stem cells are not confined to the forebrain periventricular region and indicate that stem cells endowed with different functional characteristics occur at different levels of the SVZ-rostral extension (RE) pathway. Given the persistence of neurogenesis into adulthood, several attempts have been made to identify adult NSCs. Dr. van der Kooy and his colleagues reported the possible presence of stem cells in the SVZ facing the lateral ventricle in the adult brain, using various methods including the neurosphere method.[50] Several years ago, we showed in a collaborative study with Dr. Steven Goldman, that NSCs/neuronal progenitor cells are present in adult human

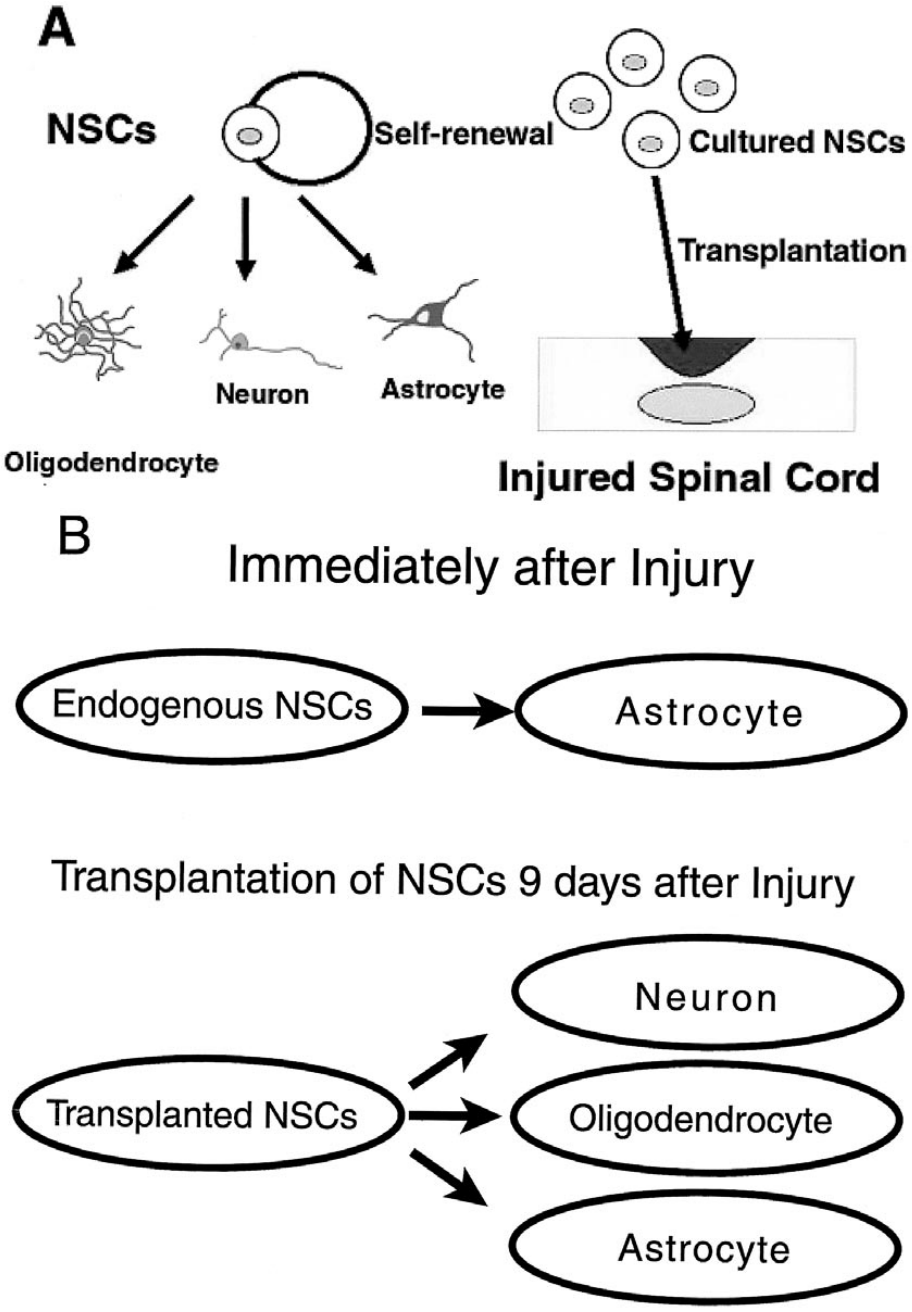

Fig. 7 Delayed NSC-transplantation in injured spinal cord. A) Scheme for therapeutic transplantation of cultured NSCs into the injured spinal cord. B) The advantage of the delayed NSCs-transplantation. The adult spinal cord immediately after injury is likely to present non-neurogenic environment.[48] However, delayed transplantation of NSCs 9 days after injury was shown to result in generation of neurons, oligodendrocytes and astrocytes from donor cells. The illustrations in this figure were provided by Dr. Yuto Ogawa (Department of Orthopedic Surgery, School of Medicine, Keio University).

brain tissues at the region facing the lateral ventricle.[51] These cells in the SVZ/ependymal cell layer facing the lateral ventricle, including Musashi1-positive cells, divided then differentiated into neurons in culture in the continuous presence of FGF-2 and BDNF. This finding shows the presence of NSCs or neuronal progenitor cells in the adult brain that have the ability to generate new neurons upon their exposure to a specific environment. Recently, more detailed studies to determine the origin of adult NSCs have been performed by two groups, although they remain controversial. Johansson *et al.* labeled cells using viruses carrying the *lacZ* gene

and the liposoluble biological dye, DiI, and concluded that ependymal cells in the ventricle are NSCs.[48] On the other hand, Dr. Alvarez-Buylla and his colleagues recently used glial fibrillary acidic protein (GFAP), an astrocyte marker, and reported that GFAP-positive cells located in the SVZ are NSCs, at least in the periphery of the lateral ventricle.[47] Using transgenic mice expressing a retrovirus receptor under the control of a GFAP promoter, they demonstrated that the GFAP-positive cells in the SVZ gave rise to new neurons and, thus, concluded that SVZ astrocytes are NSCs.[47] Whether ependymal cells or SVZ astrocytes are NSCs, it is of interest that a cell population previously considered to be glial cells includes NSCs and is related to neurogenesis *in vivo*. Anti-Musashi1 antibodies recognize not only ependymal cells and SVZ neural progenitor cells but also some astrocytes in adults (Fig. 5).[11]

The subventricular layer/ependymal cell layer facing the lateral ventricle, where NSCs are localized, corresponds to the neurogenic site in the adult brain. However, it is of more interest that NSCs are present even in non-neurogenic regions (reviewed by Temple and Alvarez-Buylla[44]). For example, NSCs were shown to exist in the ependymal layers surrounding the central canal of the adult spinal cord, where neurogenesis does not occur.[48,52] However, the NSCs in this site expressed *nestin*-EGFP (Fig. 6), formed neurospheres, and generated new neurons in culture. Thus, neurogenesis from these NSCs is likely to be inhibited *in vivo*, particularly by local environmental factors. Even after spinal cord injury, neurogenesis is inhibited, although the NSCs are capable of producing astrocytes,[48,52] which form glial scarring thereafter. Given these experimental findings, it is unlikely that the simple introduction of NSCs into an injured region would improve the pathology, because the endogenous NSCs generate only glial cells and not neurons. Thus, in addition to the transplantation of NSCs, preparation of the microenvironment to induce neurogenesis would be essential. We recently demonstrated that delaying the transplantation of NSCs to 9 days after the spinal cord injury, when inhibitory cues that prevent neurogenesis are likely to be down-regulated,[53] is important to achieve neurogenesis from the transplanted NSCs and functional recovery (Fig. 7).[54]

Neurogenesis in Adults and Its Significance

The long-held belief that neurogenesis does not occur in the adult brain of higher animals has been disproved. The concept that neurogenesis can occur in the adult mouse brain was actually proposed by Altman and colleagues. 40 years ago.[55] In the 1980's, Dr. Fernando Nottebohm and his colleagues proposed that in adult male canaries, neurogenesis occurs in the breeding season and replaces old and lost neurons in the high vocal center (HVC) in the neostriatum and that this process is closely related to learning a new song.[56–57]

As described in the preceding chapter, in rodents, neurogenesis occurs in limited regions in adults: the olfactory bulb and the hippocampal dentate gyrus. Dr. Fred Gage and his colleagues demonstrated that neurogenesis also occurs in adult humans.[58] These researchers took advantage of the fact that bromodeoxyuridine (BrdU), which labels cells at the proliferative stage, is administered to cancer patients for clinical diagnosis. Examination of the hippocampal tissue obtained post mortem from five patients revealed many BrdU-labeled neurons, demonstrating that these neurons were generated after BrdU administration and before death (16–781 days).[58]

The presence of NSCs with both pluripotency and the ability to self-renew in adults has been shown by neurosphere culture.[7,18,50] However, the sites of neurogenesis *in vivo* are limited in adults. Analysis of the physiological significance of this finding is underway, focusing on NSCs involved in neurogenesis *in vivo*. It has been reported that experiencing of a 'desirable environment' increases adult neurogenesis (granule cells) by 15% in the hippocampaldentate gyrus in mice, and also increases the efficiency of space learning performance (reviewed by Kemperman and Gage[59]). Recent studies have shown that newly generated neurons are physiologically active and are able to form new synapses with older neurons.[60] A fascinating thought, that ''an additional 15% of neurons increased learning efficiency'' (reviewed by Kemperman and Gage[59]), may be proposed, but how new neurons form a neuronal network with existing neurons and whether the new neurons are really involved in learning have yet to be determined. Interestingly, it has been shown that inversely, a stressful experience markedly inhibits neurogenesis. It is proposed that stress-induced decreases in neurogenesis within the hippocampal dentate gyrus are an important causal factor in precipitating episodes of depression. Reciprocally, it is speculated that therapeutic interventions for depression that increase serotonergic neurotransmission act at least in part by augmenting dentate gyrus neurogenesis.[61]

The reported results on adult neurogenesis in the neocortex have been controversial.[62–64] claimed that neurogenesis occurs in the prefrontal area of the frontal lobe, the lower temporal association area, and the posterior parietal association area in the cerebral neocortical association area of adult macaque monkeys. These regions are associated with higher functions such as emotion, memory, and judgment. The detection of neurogenesis in this area attracted attention because it disproved previous beliefs and provided profound implications for understanding the cellular mechanisms

of higher functions. However, Dr. Pasko Rakic and his colleagues' more recent results do not support the presence of neurogenesis in normal adult primate cortex.[64] In contrast to the results of Dr. Gould's group, Drs. Kornack and Rakic showed that, although numerous BrdU-labeled cells were distributed throughout the cerebral wall, including the neocortex, these were identified as non-neuronal cells by careful immunohistochemical observations using confocal microscopy; evidence for newly generated neurons was limited to the hippocampus and olfactory bulb in adult macaque monkeys.[64] Interestingly, however, this condition is likely to change upon injury. We found that significant neurogenesis was induced upon ischemia at least in some part of the neocortex in adult monkeys (Tonchev AB, Yamashima T, Zhao L, Okano HJ and Okano H: submitted).

Prospective for Cell Therapy of the Damaged CNS

Regenerative medicine using stem cell biology is attracting a lot of attention. The adult mammalian CNS was traditionally considered to be lacking in regeneration ability, but it is now an important target of regenerative medicine. Strategies for regenerative therapy using a stem cell system are roughly divided into: i) the use of organ (or tissue) stem cells (*e.g.*, NSCs), ii) the use of embryonic stem (ES) cells, and iii) the use of dedifferentiation/transdifferentiation of differentiated cells. Therapeutic strategies should be determined based on a comprehensive consideration of the available information. Each of these strategies is reviewed below.

Use of tissue stem cells (*NSCs*)

The presence of characteristic stem cells (tissue stem cells) has been confirmed in many organs or tissues within a mature body. As discussed above, stem cells are self-renewing undifferentiated cells with pluripotency, and the ability to contribute to tissue repair after injury may be added to the definition. The high potential for tissue stem cells to be applied to the regenerative therapy for various organs can be predicted from these definitions. The first successful organ regeneration using the tissue stem cell system was bone marrow transplantation.[65] In addition to the hematopoietic system, there is evidence for stem cells in many organs, such as the liver, intestinal tract, skeletal muscle, mammary gland, neural crest (peripheral nervous system and smooth muscle), skin, and CNS. Indeed, NSCs are tissue stem cells of the CNS. As described above, several groups, including ours, have sought to establish a technique for the prospective identification of NSCs.[17,24,25] The prospective identification and isolation of stem cells from organs by combining cell surface antigens or specific reporter genes with cell separation using a cell sorter is an important technique. Stem cells of each organ will eventually become commercially available as promising therapeutics for tissue repair and organ regeneration.

Regeneration of an organ by activation of the tissue stem cells within it is also an important direction of study, which may show a lot of achievement in the future, because this is considered a particularly important therapeutic strategy for regeneration of the CNS. Notably, recent findings have raised the possibility that the brain has a latent capacity for self-repair in response to injury or disease through the use of the endogenous NSCs or neural progenitor cells.[66] Dr. Jeffery Macklis and his colleagues demonstrated that the endogenous NSCs (or neural progenitor cells) can be induced *in situ* to differentiate into cortical projection neurons in regions of the adult mammalian neocortex that do not normally undergo any neurogenesis. Furthermore, these neurons form appropriate connections after chromophore-targeted synchronous apoptotic degeneration of neurons is induced.[66] These results indicate that neuronal replacement therapies for neurodegenerative disease and CNS injury may be possible through the manipulation of endogenous NSCs *in situ*. Furthermore, recent results indicate that exogenous BDNF supplied either by infusion of the recombinant protein,[67] or by the intraventricular delivery of and ependymal infection by adenoviral vectors,[68] induced neurogenesis from resident progenitor cells in the adult brain, even at sites where adult neurogenesis does not occur normally. Thus, it is likely that the adult brain parenchyma may recruit and/or generate new neurons, which could replace those lost as a result of injury or disease.

In addition to the activation of endogenous tissue stem cells, another approach for regenerative medicine using tissue stem cells is, of course, their transplantation, as was first performed for reconstruction of the hematopoietic system.[65] There are some promising reasons to pursue NSC-transplantation as a treatment for CNS damage. The effects of transplanting NSCs in an attempt to obtain functional recovery from various types of CNS damage have been reported recently.[4,19,69–74] For Parkinson's disease (PD), clear evidence of dopaminergic fetal graft survival and symptomatic relief – (at least for younger patients –) has been recently provided by open label clinical trials[75] and a double-blind, sham-surgery controlled study.[76] However, 5∼10 fetal brains are required to treat one PD patient, causing practical and ethical problems with this procedure. On these grounds, there has been increasing interest in developing procedures to obtain large amounts of standardized and quality-

controlled NSCs. However, it should be noted that NSC transplantation therapy in PD is still in its infancy. Although *in-vitro*-expanded human NSCs (neurospheres) derived from the fetal cortex can survive for long periods, differentiate into neurons and astrocytes, and extend long processes into the host brain, dopaminergic (DA) neurons do not arise very often *in vitro* or following transplantation.[77,78] On the other hand, NSC-transplantation still holds much promise for some cases. Studies have demonstrated that the fates of the transplanted NSCs or progenitor cells greatly depend on the host microenvironment, *e.g.*, the timing of the transplantation,[54,79,80] the transplantation site (neurogenic or non-neurogenic sites), and the cell types that are lost within the host CNS upon injury or disease.[72] We transplanted rat fetal spinal cord-derived NSCs grown *in vitro* into a rat spinal cord injury model (cervical compression model) and successfully obtained histological and behavioral recovery.[54] Further study using a primate donor and recipient will yield useful information for the application of these findings to humans. The range of NSC applications aimed at regeneration of the CNS is not limited to cell therapy by transplantation. The investigation of how endogenous NSCs are activated and induced to contribute to organ self-repair are also important areas of research.

Use of embryonic stem cells (*ES cells*)

ES cells are undifferentiated stem cell lines established from the epiblasts that are present in the inner cell mass (ICM) of an early embryo at the blastocyst stage. Epiblasts differentiate into three germ layers that will later form the body in the developmental process and thus, they are stem cells that are able to differentiate into the three germ layers. ES cells can be induced to differentiate to various cells derived from the three germ layers under various culture conditions, and human ES cell lines have been established; therefore, ES cells have been a focus of regenerative medicine.[81] To date, various cell types, including those of the nervous system, fat, smooth muscle, skeletal muscle, the hematopoietic/vascular system, and cardiac muscle have been derived from ES cells.

Several studies have applied ES cells to the nervous system. When glial progenitor cells derived from mouse ES cells were injected into rat mutants showing hypomyelination,[82] the tissue was stained with an antibody against a myelin-specific protein marker, PLP. Recently, Dr. Ole Isacson and his colleagues showed that transplanting low doses of undifferentiated mouse ES cells into the striatum of a rat PD model (6-OH DA unilaterally-lesioned hemiparkinsonism) results in a proliferation of ES cells into fully differentiated DA neurons and a gradual and sustained behavioral restoration.[83] However, teratoma developed in rare instances after transplantation in these cases, indicating that improvements to this procedure still need to be designed and tested before it can be applied as a cell therapy for PD. Dr. Yoshiki Sasai and his colleagues at Kyoto University reported that DA neurons are efficiently induced *in vitro* when mouse or monkey ES cells are co-cultured with PA-6 cells (stromal cells), and that these neurons survive after their transplantation into the striata of PD model mice.[84,85] This method makes it possible to derive DA neurons from ES cells, but the derived DA neurons are intermingled with other types of cells. To apply this method to therapy, it is necessary to purify the dopaminergic neurons and to exclude other types of cells, including undifferentiated ES cells, which could form a teratoma after transplantation. We developed a reporter gene (TH-EGFP) containing cDNA encoding green fluorescence protein under the control of the promoter for tyrosine hydroxylase, the key enzyme of dopamine biosynthesis[86] and designed a system for purifying TH-positive neurons using the TH-EGFP reporter gene and cell sorter (Fig. 8). Neverthe-

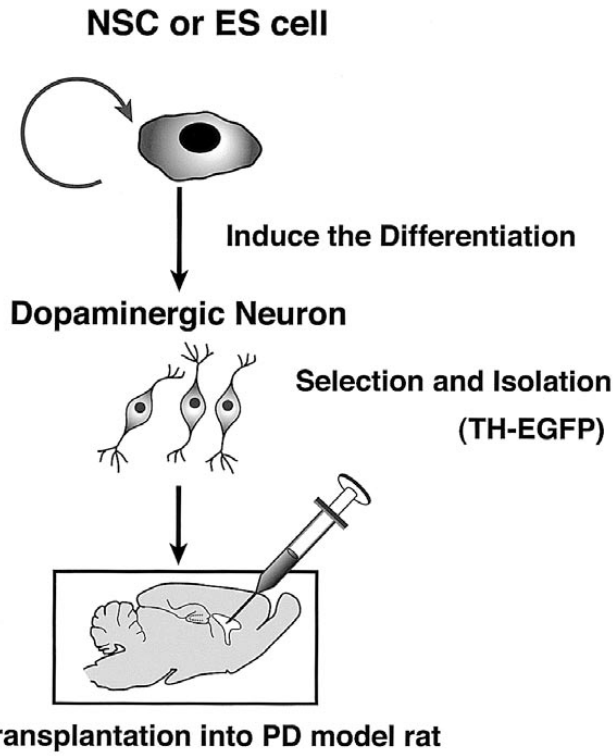

Fig. 8 A new strategy for the cell therapy of Parkinson's disease. A large quantity of dopaminergic neurons can be prepared from *in-vitro*-expanded NSCs or ES cells, followed by effective induction into dopaminergic neurons and their subsequent purification using the TH-EGFP reporter and FACS-mediated isolation. This could provide a new strategy for the cell therapy of Parkinson's disease, overcoming the ethical and practical problems associated with the current fetal grafts.

less, it is also important to investigate whether the introduced ES cell-derived dopaminergic neurons are functional *in vivo*. It was recently shown that, in many neurodegenerative diseases including PD, specific neurons are selectively lost (amyotrophic lateral atrophy [ALS], motor neurons; Huntington's chorea, striatal GABAnergic neurons; and cerebellar ataxia, cerebellar neurons including Purkinje cells). This implies that treatment of such diseases using ES technology would require a two-step strategy: efficient derivation of these neurons from ES cells and their selective isolation.[87]

As human ES cells become more available, the following points should be addressed regarding their usage in medical treatment: I) The direct transplantation of ES cells induces teratoma, as observed in immunodeficient animals such as SCID mice. It would be better to induce ES cell differentiation to a more mature state, *i.e.*, more like the cells constituting the body, then transplant them, rather than to transplant them directly. II) Even when cells are induced to differentiate, various cell types are derived in the differentiation via the formation of the embryoid body, and separation of the specific cells required for the therapy is necessary. III) The possibility of immunological rejection always exists in transplantation therapy, except with autografts. It is theoretically possible to perform therapeutic cloning, in which human ES cell pools of each HLA type are prepared or nuclear transferred ES cells are generated (ntES cells) by denucleating an unfertilized oocyte, injecting the somatic cell nucleus of a specific person into the embryo, culturing the embryo until the blastocyst stage, and establishing ES cells of the specific person from the inner cell mass of the cloned embryo. The preparation of a human embryonic clone reported by a US venture business group, Advanced Cell Technology Co. and was sparked widespread debate about the ethics of this research.[88] The human embryonic clone prepared by this group divided only to the 6-cell stage and embryogenesis did not reach the blastocyst stage, which is thought to be required for the establishment of ES cells. However, when they induced the parthenogenesis of an unfertilized oocyte that had completed the second meiosis, using cytochalasin B treatment and a calcium ionophore, the embryogenesis progressed to the blastocyst stage. Notably, ES cells were recently established from non-human primate parthenogenic blastocysts (termed, ''parthenogenic stem cells'', PS cells).[89] It is still unclear whether PS cells can be used as immune-rejection-free donor cells in regenerative therapy, because the consequences of the homozygosity of recessive mutations in these cells are unknown.

The implications of these recently developed ES cell technologies in therapeutic applications have only begun to be discussed. It is critical to discuss the ethical issues associated with studies using human ES cells, which are complex, and attitudes vary among countries.

Use of dedifferentiation/transdifferentiation

Tissue stem cells have been found in various organs, and these stem cells supplement differentiated cells lost due to physiological turnover or injury. Accordingly, until a few years ago, it was commonly thought that endogenous tissue stem cells in each organ only generate cells that are specific to that organ. However, this view was challenged by one study in which the differentiation and proliferation of hematopoietic cells were derived from NSCs.[90] Although there has been some difficulty reproducing this particular result,[91] since this report, other studies on the reprogramming, or ''plasticity,'' including transdifferentiation and dedifferentiation of mammalian cells have appeared in the literature. These recent studies include: I) the transdifferentiation of bone-marrow-derived cells into neural cells,[92–95] II) the transdifferentiation between muscle- and bone-marrow-derived cells,[96,97] III) the transdifferentiation of bone marrow cells[98,99] or purified hematopoietic stem cells[100] into liver cells; IV) the differentiation of purified NSCs injected into a blastocyst into various cells other than nervous system cells,[101] V) the conversion of NSCs to skeletal muscle upon co-culturing,[102] VI) the dedifferentiation of oligodendrocyte progenitor cells into NSCs under certain culture conditions,[103] VII) the differentiation of skin cells (dermis) into neurons, glial cells, and fat cells,[104] VIII) the differentiation of cord blood cells into neural cells,[105] and the multiorgan and multilineage potential of a single bone-marrow-derived stem cell upon engraftment.[106]

These findings indicate that the ability of stem cells to differentiate, which was thought to be tissue-specific, is unexpectedly flexible. It has been proposed that a few pluripotential cells like ES cells exist in each organ in a mature body and that such cells differentiate into specific cells under specific conditions.[107] However, some studies reported in the last few years have been difficult to reproduce. Unfortunately, the study reporting that NSC-derived blood was obtained from a mouse that had received an intravenous injection of NSCs from another mouse has not been confirmed by an additional study. The latest thinking is that the phenomenon that was considered to be the plasticity of stem cells is more likely to result from the fusion of transplanted cells and host cells.[108,109] Previous studies reporting the conversion of transplanted bone marrow cells into unexpected cell types *in vivo*[92,93,96,98–100,106] based the apparent transdifferentiation on the presence of a donor-derived marker (*i.e.*, the Y chromosome when the donor is male and the recipient is female or the expression of a donor-specific gene) in cells that had acquired a new phenotype within the recipient animals. However, a recent

report by Terada *et al.*[108] raises the alternative possibility that donor bone marrow cells can fuse with recipient somatic cells *in vivo* and that this process may contribute to some of the apparent ''donor-typed'' cells in various organs, although in this study, which characterized the phenomenon *in vitro*, the frequency of the spontaneous cell fusion was very low (2–11 clones per 10^6 bone marrow cells). Careful investigation of this issue using both donor- and recipient-derived markers will be necessary to clarify the mechanisms involved.

Although it is difficult to distinguish from reprogramming or plasticity, unexpected pluripotency has been observed in mesenchymal stem cells (MSC) in bone marrow interstitium, and skin-derived precursors (SKP) *in vitro*,[104] in which the phenotype is not likely to result from cell fusion. In addition to osteoblasts, MSCs have been shown to differentiate into hepatocytes, skeletal muscle, cardiac muscle, cartilage, fat cells, and neural cells in culture.[94,95,110–112] From SKP, neurons, astrocytes, oligodendrocytes, fat cells, and smooth muscle cells were differentiated.[104] Although many issues remain to be resolved, such as whether differentiation can cross germ layers and the difficulty in distinguishing MSC's diversity from pluripotency, it is likely that we will see the day when MSCs can be recovered by bone marrow puncture at an outpatient clinic and applied in autografts for the treatment of various organ deficiencies.

Perspectives for the Future

Regenerative therapies that take advantage of stem cell technology for CNS damage including neurological diseases hold tremendous hope for the future. Although most studies aimed at the realization of these therapies are still at the basic stage, researchers worldwide, driven by the high competition in this field, may begin focusing more on clinical application. Obviously, it is important to approach this goal in a step-by-step manner, following animal experiments using progressively modified technology with studies in small numbers of patients that apply well validated assessment protocols. Many achievements in basic science have been made in Western countries, mainly the United States, but the United States has not always occupied a leading position, as it did in the genome project. Nonetheless, the United States is investing 100 million dollars in ES cell research and 200 million dollars in somatic stem cell research and development from the federal budget in the next financial year (2002), which will accelerate the research. In Japan, before medical applications of the three strategies described above can be realized, guidelines for stem-cell therapy, quality control systems to confirm the efficacy and safety of the procedures, and once efficacy and safety are confirmed, a system by which groundbreaking medical trials in a small number of patients with informed consent can take place, must be established.

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