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ORIGINAL ARTICLE

Embryonic and Adult Stem Cells As a Source for Cell Therapy in Parkinson's Disease

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Abstract

The rationale behind the use of cells as therapeutic modalities for neurodegenerative diseases in general, and in Parkinson's disease (PD) in particular, is that they will improve patient's functioning by replacing the damaged cell population. It is reasoned that these cells will survive, grow neurites, establish functional synapses, integrate best and durably with the host tissue mainly in the striatum, renew the impaired wiring, and lead to meaningful clinical improvement. To increase the generation of dopamine, researchers have already transplanted non-neuronal cells, without any genetic manipulation or after introduction of genes such as tyrosine hydroxylase, in animal models of PD. Because these cells were not of neuronal origin, they developed without control, did not integrate well into the brain parenchyma, and their survival rates were low. Clinical experiments using cell transplantation as a therapy for PD have been conducted since the 1980s. Most of these experiments used fetal dopaminergic cells originating in the ventral mesencephalic tissue obtained from fetuses. Although it was shown that the transplanted cells survived and some patients benefited from this treatment, others suffered from severe dyskinesia, probably caused by the graft's excessive and uncontrolled production and release of dopamine. It is now recognized that cell-replacement strategy will be effective in PD only if the transplanted cells have the same abilities, such as dopamine synthesis and control release, reuptake, and metabolizing dopamine, as the original dopaminergic neurons. Recent studies on embryonic and adult stem cells have demonstrated that cells are able to both self-renew and produce differentiated tissues, including dopaminergic neurons. These new methods offer real hope for tissue replacement in a wide range of diseases, especially PD. In this review we summarize the evidence of dopaminergic neuron generation from embryonic and adult stem cells, and discuss their application for cell therapy in PD.

Index Entries: Bone marrow stromal cells; dopamine; dopaminergic neurons; Parkinson's disease; stem cells.

Introduction

Parkinson's disease (PD) is a well-characterized disease of the central nervous system (CNS). The disease affects about 2% of the population over 50 yr of age. The extrapyramidal signs and symptoms of PD (resting tremor, rigidity, hypokinesia, bradykinesia, etc.) result mainly from the degeneration and loss of dopaminergic neurons of the nigrostriatal pathway. Current treatment regimens for PD consist primarily of pharmacological supplementation of the dopaminergic loss with dopamine (DA) agonist and levodopa (L-3-4-dihydroxyphenylalanine), a precursor of DA. Levodopa, which can readily cross the blood–brain barrier (BBB), is the most effective agent controlling the symptoms of PD. Most

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Parkinson's patients have a good initial response to levodopa, but after a few years become subject to adverse effects, which include dyskinesia, fluctuations of efficacy (on–off effect), freezing, mental changes, and loss of efficacy.

Functional replacement of specific neuronal populations through transplantation of neural tissue represents an attractive therapeutic strategy for PD. In the late 1970s, Andrew Bjorklund and collaborators demonstrated that the transplantation of embryonic DA neural tissue, obtained from the fetal ventral mesencephalon, could reverse the symptoms of DA depletion in the unilateral 6-hydroxydopamine (6-OHDA)-treated rat model of PD (Bjorklund and Stenevi, 1979; Brundin et al., 1988). Encouraged by these findings in animal models, Olle Lindvall and collaborators, launched a clinical program in 1984–1985 to attempt transplantation of embryonic neural tissues into the brains of PD patients. Clinical trials with transplantation of human embryonic mesencephalic tissue into the caudate and putamen (striatum) of PD patients were initiated in 1987, and about 350 patients have since undergone transplantation (Lindvall and Hagell, 2002a). These clinical tests showed that grafts of fetal ventral mesencephalon successfully survive and reduce motor symptoms (Sauer and Brundin, 1991; Frodl et al., 1994; Lindvall, 1994; Nakao et al., 1994; Haque et al., 1997; Bjorklund and Lindvall, 2000; Okano et al., 2002). Although transplantation is a promising treatment for PD, it requires brains from about 5–10 fetuses for even one PD patient, thus causing ethical and practical problems, limiting its clinical application. The mammalian adult brain is a very plastic system that is capable of incorporating transplanted stem cells into functional neurotransmission. In recent years the questionable benefit and safety of this procedure was raised as a control study pointing to the high risk of adverse signs such as tardive dyskenesis (Freed et al., 2001, Freed, 2002; Olanow et al., 2003). The challenge of cell replacement in PD is huge, and efforts to find the best cell source are still being continued with high priority. To overcome this problem, researchers are turning to stem cell biology for materials to use in the therapeutic transplantation of PD. Many researchers have investigated the use of a wide variety of candidate cells as possible transplantation donor cells for PD therapy. Our group has investigated bone marrow stromal cells (BMSCs) for experimental therapeutics in PD model animals (Levy et al., 2003a,b). However, the complete and coordinated induction of specific neuronal phenotype in multipotent neural precursors in vitro has proved elusive. The initial success of levodopa treatment for PD suggested the feasibility of DA replacement therapy by neural transplantation; and the small size of striatum (or caudate putamen in human beings), which becomes DA denervated in PD, makes it an easily accessible target for transplantation (Freeman et al., 1998). Transplantation of DA-producing tissue has received considerable attention as an alternative therapy that delivers DA directly to the striatum, sparing other tissues from adverse effects of DA stimulation and metabolism, and avoiding the drug peaks and valleys of pharmacological administration by providing a relatively constant source. Much of the scientific efforts during the past 15 yr have therefore had to provide proof of principle that (1) the grafted DA neurons can survive and form connections in the PD patient's brain, (2) the patient's brain can integrate and use the grafted neurons, and (3) the grafts can induce a measurable clinical improvement (Lindvall, 2003).

Stem cells have the remarkable ability to exist in vivo in a dormant, undifferentiated state and to self-propagate. Stem cells are not restricted to cell types specific to the tissue of origin, and so they are able to differentiate in response to local environmental clues from other tissues. This capability of self-renewal and differentiation has great therapeutic potential in curing diseases. The ideal stem cells are present in the embryo, although recent studies point to various sources of stem cells in several organs and mature tissues. Although stem cells possess great promise as a cure for diseases of the nervous system, methods of enriching and producing functional cells must be developed. Cell-replacement strategy will be effective in PD only if the transplanted cell has the same abilities, such as DA synthesis and control release, reuptake and metabolizing DA, as the original DA neurons.

Embryonic stem (ES) cells are pluripotent cells isolated from the inner cell mass, a cluster of a few hundred identical cells in the blastocyte, which is formed in the early stage of embryonic development. ES cells can proliferate extensively in an undifferentiated state and can provide an unlimited source of many tissue types. One advantage of using ES cells is their accessibility to genetic engineering, which permits the isolation and functional analysis of specific cell types (Kim et al., 2002). The use and growth of ES cells are not without disadvantages, and the use of the fertilized egg raises ethical concerns and limitations. Moreover, culture of these cells is complicated and needs

supplementing. In addition, their further clinical use is restricted, owing to their possible formation of tumor-like masses, called teratoma.

In recent years, it has been found that cells derived from mature tissue can be reprogrammed and can display a completely distinct phenotype from that found normally. Mature tissues have also a small number of pluripotent stem cells with a greater differentiation potential (Asahara et al., 2000). It has been suggested that in the brain, or other organs, the environment might encourage survival and differentiation of cells for the appropriate destination. BMSCs, also referred to as mesenchymal stem and progenitor cells, give rise to cells of mesenchymal lineage such as osteoblasts, cartilage, and muscle (Prockop 1997). BMSCs secrete a substantial amount of growth factors and cytokines into the soluble stroma providing a supportive microenvironment for both the growth and differentiation of hematopoietic stem cells. In this review, we will discuss at length the potential of BMSCs to differentiate into dopaminergic neuron cells.

The use of in vitro expanded fetal tissue precursors poses the same ethical, logistic, and technical problems of transplantation in neurodegenerative disease. Hence, stem cells from fetal mesencephalic precursors, or from a fetal source, are not discussed in this review.

The aim of this article is to describe and illustrate current research strategies for generating tyrosine hydroxylase (TH) cells and/or DA neurons from embryonic and adult stem cells, and to discuss the possible role of this technology to further develop cell-replacement therapy in PD.

Stem Cell

The burgeoning field of stem cell biology has attracted considerable interest over the last decade, in part, because of the unique properties of stem cells that potentially make them an ideal solution to the problems of tissue supply for cell-replacement therapies, such as PD. A stem cell can be defined as a cell that is capable of self-renewal and multilineage differentiation. In practice, this means that these cells undergo asymmetric division to yield other stem cells and non-self-renewing progenitor cells with a more restricted differentiation potential. Stem cells have the remarkable potential to develop into many different cell types. Serving as a sort of repair system for the body, they can theoretically divide limitlessly to replenish other cells for life's duration. When a stem cell divides, each new cell remains a stem cell or can become another type of cell with a more specialized function, such as a muscle, red blood, or brain cell. The archetypal stem cell, and that which is formed earliest in development, is the ES cell. These totipotent cells can be isolated from the inner cell mass of the blastocyst or from primordial cells of the germ cell lineage. Later in development, the tissue-specific stem cells that are part of a specific organ system, and under physiological conditions, differentiate only into phenotypes appropriate for that tissue. Several studies have shown that exposure of stem cells, embryonic or adult, to specific transcription factors induce differentiation to dopaminergic neurons (Fig. 1).

From Stem Cell to Dopaminergic Neuron

There are two techniques of using stem cells for grafting in PD. Initially, cells are differentiated in vitro to dopaminergic neurons prior to transplantation. Thus, stem cells are a potentially unlimited source for the generation of DA neurons. Cell preparations could be standardized and quality-controlled with respect to viability and purity. Second, stem or progenitor cells are differentiated in vivo to dopaminergic neurons after implantation into the striatum or substantia nigra (SN). These neurons might integrate better, as compared with primary embryonic DA neurons and, in the ideal situation, reconstruct the nigrostriatal pathway. To make this possible, however, the mechanisms instructing the immature stem or progenitor cells to differentiate into the missing DA neurons must also operate in the PD patient's brain (Lindvall and Hagell, 2002b; Lindvall, 2003).

Hypothetically, DA neurons could be produced from stem cells of four different sources: ES cells from the fertilized egg, neural stem cells from the embryonic or adult brain, or stem cells of other tissues. The crucial question remains whether the generated neurons become functional DA neurons. Another still unresolved issue is whether non-dopaminergic neurons and glial cells, normally present in the mesencephalic grafts used so far in PD patients, are important for the differentiation and functioning of DA neurons. In this case, an enriched population of predifferentiated DA neurons might not be the optimal preparation (Lindvall and Hagell, 2002b; Lindvall, 2003).

Of these methodologies, the second revolution in the understanding of treatment options for PD (the

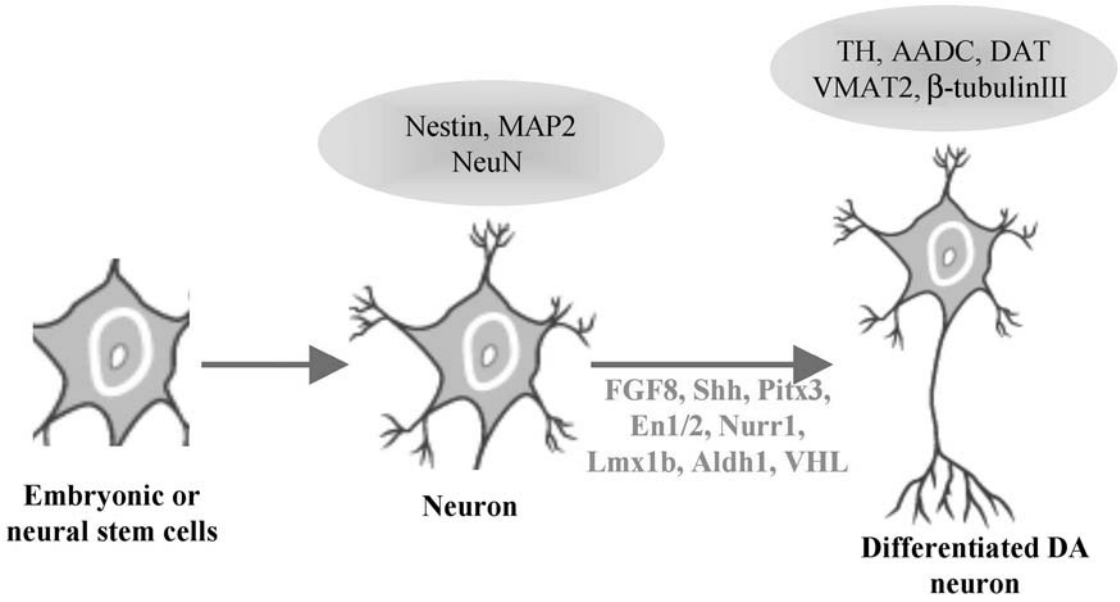

Fig. 1. Midbrain DA neuronal differentiation from ES cells or NSCs. Illustration of developmental factors involved in the classification and production of midbrain DA neurons from mouse ES cells. Neuronal nuclear antigen (NeuN), MAP2, or nestin, the intermediate filament of neuronal progenitor cells, is a typical neuronal marker. Midbrain DA neurons are generated at the junction of midbrain and hindbrain in response to a ventral-dorsal gradient of floor plate-derived Shh and an anterior-posterior gradient of FGF-8. The Lmx1b and Nurr-1 transcription factors are essential for different aspects of DA differentiation. The Pitx3, En1/2 transcription factor, von Hippel-Lindau tumor suppressor (VHL), and the retinoid-synthesizing enzyme, Aldh1, are specific markers of developing DA neurons in the ventral midbrain, but their roles are still unknown. Midbrain DA neurons can be identified by the expression TH, L-aromatic amino acid decarboxylase (AADC), DAT, the vesicular monoamine transporter 2 (Vmat2), and the neuronal marker β-tubulin III.

first being the discovery of levodopa) is neural cell transplantation, which has opened up a completely new avenue for brain repair. Instead of chemically controlling a degenerated system, this method allows reconstruction and renovation of the DA system by new sets of cells performing the normal function of those destroyed (Isacson, 2002).

Stem cell biology related to brain development and repair can be approached by methods using either adult or ES cells potentially capable of generating new neurons, after selective expansion in cell-culture systems or in the living brain.

The possibility of generating DA neurons has been explored in several approaches using stem cells from different sources.

ES Cells As a Source for Dopaminergic Neurons

Rodent ES Cells

The last few years have witnessed a burgeoning of studies defining in vitro requirements for guiding ES cells into specific cell lineages. There are at least three fundamental strategies for inducing neural differentiation of mouse ES cells. The classic approach is based on the formation of embryoid bodies, free-floating structures of aggregated ES cells composed of the progeny of all three germ layers. Neural differentiation within embryoid bodies can be induced by exposure to retinoic acid (RA), such as in the classic 4–/4+ protocol (4 d in the absence of, followed by 4 d in the presence, of RA) (Bain et al., 1995). A second successful approach for DA neuron generation from mouse ES cells is based on the stromal-derived inducing activity (SDIA) of stromal cell lines such as PA-6 (Kawasaki et al., 2000). The third strategy for the neural differentiation of mouse ES cells is based on the neural default model of differentiation (Munoz-Sanjuan and Brivanlou, 2002). Growth of ES cells at low density, in minimal medium, and in the absence of any feeders, serum, or growth factors leads to spontaneous neural differentiation of a significant proportion of ES cells. These findings raise interesting developmental questions. However, the comparatively low efficiency, the absence of dopaminergic markers, and the lack

of functional data currently make this system less suited for cell therapeutic approaches (Perrier and Studer, 2003).

To assess the potential of ES cells to undergo neuronal differentiation in vivo, stem cells from mouse blastocysts were transplanted, with (0.5 m*M*) or without RA pretreatment, into adult mouse brain and adult lesioned rat brain (Deacon et al., 1998). Intracerebral grafts survived in 61% of cyclosporine-immunosuppressed rats and 100% of mouse hosts, exhibited variable size and morphology, and developed large numbers of cells exhibiting neuronal morphology and immunoreactivity for neurofilament, neuron-specific enolase (NSE), TH, serotonin, and cells immunoreactive for glial fibrillary acidic protein (GFAP). Though graft size and histology were variable, typical grafts of 5–10 mm^3 contained 10,000–20,000 TH^+ neurons, whereas DA-β-hydroxylase positive ($D\beta H^+$) cells were rare. Most grafts also included non-neuronal regions. In intracerebral grafts, large numbers of astrocytes immunoreactive for GFAP were present. Both TH^+ and serotonergic axons from intracerebral grafts grew into regions of the DA-lesioned host striatum. Tyrosine hydroxylase¡positive (TH^+) axons from both RA^+ and RA^- ES cell grafts extended into the DA-denervated host striatum. These TH^+ axons grew preferentially into host striatal gray matter, avoiding corticofugal white-matter tracts of striatum. In contrast to TH^+ graft axons, serotonergic axons showed no white/gray matter preference. The morphology of serotonergic neurons within these grafts also differed from TH^+ cells and axons; serotonergic neurons had less robust dendritic trees than TH^+ neurons and lacked axonal varicosities. These findings demonstrate that transplantation of these neurons to the brain can: (1) induce a significant fraction of totipotent ES cells to become putative dopaminergic or serotonergic neurons and (2) produce neurons capable of innervating the adult host striatum.

Support for the above in vivo results came from the study of Björklund et al. (2002). They have shown that undifferentiated mouse ES cells grafted in small numbers into the striatum of 6-OHDA-lesioned rat survive for 14–16 wk and develop into normal midbrain-like DA neurons that express dopaminergic and neuronal markers such as TH, neural nuclei protein (NeuN), DA transporter (DAT), and L-amino acid decarboxylase (AADC). All TH^+ neurons coexpressed calretinin, which normally is coexpressed with TH in both A9 and A10 regions of the ventral midbrain, and some TH-positive neurons coexpressed calbindin, which is found primarily in A10 DA neurons. In addition, ES cell-derived TH^+ neurons coexpress the A9 midbrain DA neuron marker aldehyde dehydrogenase 2 (Aldh2). These findings demonstrate that grafted ES cells differentiate into an adult ventral mesencephalic-like DA neuronal phenotype after transplantation in vivo at low cell densities and doses. Furthermore, differentiated ES cells developed numerous serotonergic neurons. In addition to monoaminergic neurons, grafts also contained a small number of γ-aminobutyric acid (GABA), as well as choline acetyltransferase (ChAT), neurons. Numerous astrocytes stained for the astrocyte marker GFAP within the grafts were found. When a small number of cells are used, ES cell-to-cell contact is reduced and the influence from the adult host striatum increases. Animals with ES cell-derived DA neurons recovered from amphetamine-induced turning behavior, whereas control (sham surgery) animals did not. Importantly, the decrease in rotational scores was gradual, and animals with ES cell-derived DA neurons showed a significant decrease in rotations from pretransplantation values at 7 and 9 wk. Moreover, by using animals showing behavioral recovery of rotational asymmetry at 9 wk after implantation of ES cells, researchers found an increase in carbon-11-labeled 2β-carboxy-3β-(4-fluorophenyl) tropane ([^{11}C]CFT) binding in the grafted striatum of 75–90% of the intact side was shown by positron emission tomography (PET), whereas markedly less specific activity (<25% of intact side) was found in sham controls. These results must be reviewed with caution because of teratoma formation and lack of cell survival seen in some rats (Table 1).

R. D. McKay at the National Institutes of Health (NIH) (Lee et al., 2000) described a method for generating DA neurons from ES cells to thus open the way to unlimited in vitro production of these neurons. The method involves several steps: stage 1, expansion of undifferentiated ES cells; stage 2, generation of embryoid bodies; stage 3, use of a defined medium to select for CNS stem cells; stage 4, proliferation of CNS stem cells in the presence of mitogen, basic fibroblast growth factor (bFGF, FGF2); stage 5, differentiation of stem cells by removal of mitogen. Under optimal culture conditions, 72% of ES cells assumed a neuronal morphology, 34% of neurons were dopaminergic, and 11% were serotonergic. Serotonin and TH were not coexpressed. Transplantation of these cells was not performed. Using differentiated ES cells that overexpressed the transcription factor, nuclear receptor-related 1

Table 1
In Vivo Differentiation of Stem Cells to Neural Lineages After Transplantation

Population of cells/source	Differentiation	Animal model	Dopaminergic and neuronal markers (Surviving TH^+ cells)	Behavioraral test	Behavioraral recovery	Ref.
Embryonic stem cells (ESc)						
Mouse	With or without RA	6-OHDA lesioned nigrostriatal, rat model for PD	TH (14,500), NSE, NF-H	NT	NT	Deacon et al., 1998
Mouse	Small number of undifferentiated cells	Lesioned the median forbrain bundle by 6-OHDA, rat model for PD	TH (2100), NeuN, DAT, AACD, Aldh2, calretinin, calbindin, serotonin	Rotation, PET	+	Björklund et al., 2002
Mouse, transfected with Nurr-1	Five-stage protocol	Lesioned striatum by 6-OHDA, rat model for PD	TH (NT), calbindin	Rotation, step test, paw-reaching test, cylinder test	+	Kim et al., 2002
Mouse	Differentiated using a five-stage in vitro method	6-OHDA-lesioned striatum, mouse model for PD	TH (NT)	Rotational	+	Nishimura et al., 2003
Mouse, transfected with Bcl-XL	Differentiated using a five-stage in vitro method	Lesioned SN by 6-OHDA, rat model for PD	TH (18,310)	Rotational, stepping test	+	Shim et al., 2004
Mouse	ES colonies were cultured on PA-6 cells (12 d)	6-OHDA-lesioned striatum, mouse model for PD	TH (13,000), β-tubulin III	NT	NT	Kawasaki, et al., 2000
Mouse	ES colonies were cultured on PA6 cells (12 d)	6-OHDA-lesioned striatum, mouse model for PD	TH, β-tubulin III	NT	NT	Morizane et al., 2002
Cynomolgus monkey	ES cells were cultured on PA-6 cells for 3 wk	6-OHDA-lesioned striatum, mouse model for PD	TH (830)	NT	NT	Kawasaki 2002
Mouse or nuclear transfer	5 d: co-culture on stromal cell line 2 d: SHH, FGF8, SRM 4 d: N2, SHH, FGF8 bFGF 3 d: N2, ascorbic acid, BDNF	6-OHDA lesioned striatum, mouse model for PD	TH (23,000), DAT, AADC	Rotational	+	Barberi et al., 2003

Human embryonal carcinoma cell line NT2	LiCl pretreated hNT-DA neurons	6-OHDA-lesioned nigrostriatal, rat model for PD	TH (few)	Rotational	No	Baker et al., 2000
Bone marrow stromal cells (BMSCs)						
Mouse	Undifferentiated	MPTP mouse model of PD: cells grafted in striatum 1 wk later	TH (52)	Rotarod test	+	Li et al., 2001b
Mouse	Vectors construct consisting of the GDNF gene	MPTP mouse model of PD; cells grafted intravenously 6 wk before	TH	Locomotor activity	+	Park et al., 2001
Rat BMSc	Vectors construct consisting of the TH gene and GC gene	6-OHDA rat model for PD	TH, GC, L-DOPA	Rotation	+	Schwarz et al., 1999
Neuronal stem cells (NSCs)						
c17.2 mouse	NSCs engineered to release GDNF	6-OHDA-lesioned striatum, mouse model for PD	Nestin, Neu-N, GFAP, CNPase, TH (350)	Rotation	+	ÅKerud et al., 2001
c17.2 mouse NSCs	Undifferentiated	6-OHDA-lesioned striatum, rat model for PD	β-tubulin III, NSE, NeuN, TH, AADC	Rotation	No	Yang et al., 2002
Evidence for neurogenesis in adult mice SN	Not relevant	MPTP mouse model of PD	TH, Hu, NeuN, nestin, CRMP-4	Not relevant	Not relevant	Zhao et al., 2003b
Human	Differentiation medium (not described)	Human PD	Not relevant	UPDRS	+	Levesque and Neuman, 2002a, 2000b

UPDRS, Unified Parkinson's Disease Rating Scale; *See* Table 2 for definitions. MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; 6-OHDA, 6-hydroxy dopamine; SN, substantia nigra.

(Nurr-1), they demonstrated that cells could efficiently generate functional midbrain precursors and DA neurons (Kim et al., 2002). This was shown by anatomical, neurochemical, electrophysiological, and behavioral examination. Thus, overexpression with Nurr-1 dramatically increased the proportion of TH^+ neurons. When cells were treated with FGF8 and Sonic hedgehog (Shh), the yield of cells with a dopaminergic phenotype, that is, expressing dopaminergic markers (such as TH and DAT), was increased in vitro. Moreover, depolarization markedly elevated the DA released in the cultured Nurr-1 ES cells compared with wild-type ES cells. The presence of noradrenaline and adrenaline was not observed in these cultures. Gene expression of DβH and the transcription factor Phox2a, selective markers for adrenaline-mediated neurons, was not detected. Animals grafted with wild-type ES cells showed a slight recovery in rotation behavior. However, the group grafted with Nurr-1 ES cells showed a marked change in this parameter, leading to consistent contralateral turning. The transplanted cells survived and reduced PD symptoms. No teratomas were observed in animals that had received grafts of Nurr-1 ES cells. Nuclear receptor-related 1 (Nurr-1) ES cells also developed functional synapses and demonstrated electrophysiological properties typical of mesencephalic neurons. Behavioral tests for recovery, such as amphetamine-stimulated rotations, paw-reaching test, and cylinder test showed that the group grafted with Nurr-1 ES cells demonstrated marked improvement. However, it is as yet unknown if the cells give rise to a functional reinnervation of the striatum, and their efficacy compared with primary embryonic DA neurons. Finally, additional questions are of long-term safety and whether this type of genetic modification of ES cells is acceptable in a clinical setting (Lindvall, 2003).

According to their study, Nishimura et al. (2003) used differentiated ES cells in a mice model of PD induced by 6-OHDA. Undifferentiated ES cells carrying the enhanced green fluorescent protein (GFP) gene were differentiated into a cell population containing TH^+ neurons using a five-step in vitro method. These differentiation ES cell-derived cells were used as allografts in parkinsonian mice after administration of 6-OHDA injections. Tyrosine hydroxylase-positive ($TH^{+)}$ cells were found at the grafted sites 8 wk after transplantation, some of which were immunopositive to GFP, demonstrating the presence of dopaminergic neurons derived from ES cells.

Shim et al. (2004) generated mouse ES cells that constitutively express Bcl-XL, an anti-apoptotic protein of the Bcl-2 family. In vitro differentiation, as described previously, resulted in higher expression of genes relating to midbrain DA neuron development and increased the number of ES cell-derived neurons expressing midbrain DA markers (TH^+, 31%), compared with the differentiation of wild-type ES cells (TH^+, 18%). Similarly, serotonin cells were increased from 9.4% in control ES cells to 15% in Bcl ES cells. This suggests that Bcl-XL enhances overall midbrain DA neuron differentiation and is not limited to TH expression. Moreover, DA neurons derived from Bcl ES cells were less susceptible to 1-methyl-4-phenylpyridium, a neurotoxin for DA neurons. When transplanted into parkinsonian rats, the Bcl ES cell-derived DA neurons exhibited more extensive fiber outgrowth and led to a more pronounced reversal of behavioral symptoms than wild-type ES-derived DA neurons.

Higher levels of neural differentiation are achieved by treatment of aggregates with RA in the presence of serum (Bain et al., 1995) or by coculture with a particular stromal cell line, PA-6 (Kawasaki et al., 2000). The action of RA is pleiotropic and of indeterminate physiological relevance, whereas the effect of PA-6 cells is attributed to an undefined neural inducing activity, SDIA.

Existing protocols for the neural differentiation of mouse ES cells require extended in vitro culture, yield variable differentiation results, or are limited to the generation of selected neural subtypes. Studer and colleagues (Barberi et al., 2003) improved techniques for in vitro differentiation of mouse ES cells into several subtypes by providing a set of coculture conditions that allow rapid and efficient derivation of most CNS phenotypes. The fate of both fertilization- and nuclear transfer-derived mouse ES cells was directed selectively into neural stem cells, astrocytes, oligodendrocytes, or neurons. Specific differentiation into synthesized GABA, DA, serotonin, or motor neurons was achieved by defining conditions to induce forebrain, midbrain, hindbrain, and spinal cord identity. The conditions induced neural differentiation by coculturing ES cells with murine bone marrow-derived stromal feeder cell (BMSC) lines. After 5 d of coculturing ES cells on a stromal cell line, Shh and FGF8 were added to the serum replacement medium, and the medium was changed to N2 supplemented with Shh and FGF8 in the presence of bFGF (d 8–11). At d 11, terminal differentiation was induced by withdrawal of Shh, FGF8, and bFGF

and the addition of ascorbic acid and brain-derived neurotrophic factor (BDNF). These cells expressed TH and the midbrain-specific transcription factors engrailed 1 (En1), LIM homeobox transcription factor 1b (Lmx 1b), Ptx3, and Nurr-1, as well as the dopamine transporter. Compared with earlier techniques, this system exhibited minimal variability in obtaining neural cells from a wide range of fertilization cell-derived cells. Neuronal function of ES cell-derived dopaminergic neurons was shown in vitro by electron microscopy, measurement of neurotransmitter release, and intracellular recording. Furthermore, in vivo survival of ES cell-derived dopaminergic neurons (Kawasaki et al., 2000) and correction of the phenotype in parkinsonian rats, using ES cells overexpressing Nurr-1, have been described (Kim et al., 2002). However, the therapeutic potential of ES cells not overexpressing Nurr-1 has not been demonstrated. In this study it was shown that transplantation of ES cell-derived dopaminergic neurons into parkinsonian mice resulted in the long-term survival of transplanted dopaminergic neurons. Transplantation of dopaminergic neurons derived from fertilized ES cells resulted in >70% correction, according to a functional test that measures chemically induced rotational behavior, demonstrating an in vivo application of therapeutic cloning in neural disease (Table 1).

Primate ES Cells

ES cells derived from primate (human and monkey) blastocytes possess a number of characteristics, such as surface antigens, leukemia inhibitory factor independence, and long doubling times, distinct from mouse cells (Thomson and Marshall, 1998; Reubinoff et al., 2000). Various studies have described the potential of human ES cells to differentiate into multiple lineages (Schuldiner et al., 2000; Odorico et al., 2001), such as neural progenitors (Carpenter et al., 2001; Reubinoff et al., 2001; Schuldiner et al., 2001), hematopoietic precursors (Kaufman et al., 2001), and insulin-secreting cells (Assady et al., 2001). One obvious goal was used to manipulate the differentiation of human ES cells so that a uniform population of precursors or fully differentiated cells could be obtained in vivo or in vitro. It was demonstrated that the adrenal marker—DβH, and brain marker—neurofilament heavy chain (NF-H) mRNA were expressed only when human ES cells, as embryoid bodies, were treated with RA (Schuldiner et al., 2000). In addition, it was found that human embryoid bodies treated with a high concentration of RA (1 μ*M*) and consequent plating caused an increase in number of embryoid bodies containing NF-H$^+$ neurons to 76% and complex network morphology. mRNA of DA receptor D1 (DRD1) and two serotonin receptors ($5HT_{2A}$ and $5HT_{5A}$) were detected in embryoid bodies following RA treatment but not in naive ES cells. Moreover, RA induced the mRNA expression of AADC, a key enzyme in the synthesis of both serotonin and DA (Schuldiner et al., 2001).

Differentiation protocols in primate ES cells to neuron are based on the concepts developed for mouse ES cell differentiation. Multistage embryoid body-based differentiation protocols for human ES cells have been reported to achieve efficient derivation of neuronal and astrocytic fates (Carpenter et al., 2001; Zhang et al., 2001). Carpenter et al. (2001) described that human ES cells were maintained for >6 mo in vitro (>100 population duplications) before their ability to differentiate into the neural lineage was evaluated. Differentiation was induced by the formation of embryoid bodies that were subsequently plated onto appropriate substrates in defined medium containing mitogens. These populations contained cells that showed positive immunoreactivity to nestin, polysialylated neural cell adhesion molecule (PS-NCAM), and A2B5. After further maturation, these cells expressed additional neuron-specific antigens (such as microtubule-associated protein 2 [MAP2], and synaptophysin) and TH, the DA rate-limiting enzyme. In addition, calcium imaging demonstrated that these cells responded to neurotransmitter application. Electrophysiological analyses showed that cell membranes contained voltage-dependent channels and that action potential was triggered by current injection. Polysialylated neural cell adhesion molecule (PS-NCAM)and A2B5 immunoselection or culture conditions could be used to produce enriched populations (60–90%), which could be differentiated further into mature neurons. The properties of human ES-derived progenitors and neurons were found to be similar to those of cells derived from primary tissue.

An alternative strategy is based on the manual lineage selection in mixed populations of differentiating human ES cells. Neural precursors are generated by default in the absence of any specific extrinsic differentiation cues. Reubinoff et al. (2000, 2001) reported the generation of enriched and expandable preparations of proliferating neural progenitors from human ES cells. The neural progenitors could differentiate in vitro into three neural lineages, astrocytes, oligo-

Table 2
In Vitro Differentiation of Stem Cells to Dopaminergic Neural Lineages

Population of cells	Induction of differentiation	Gene expression of neuronal lineage	Protein markers of neuronal lineage	Dopaminergic markers (protein/RNA)	HPLC for DA	TH (%)	Other neurotransmitter (protein/RNA)	Ref.
Rodent embryonic stem cells (ESc)								
Mouse	Five-stage protocol. Stage 4: bFGF, Shh, FGF8; Stage5: ascorbic acid	Nestin, Otx1, Otx2,	Nestin, β-tubulin III,	TH, dopamine, Nurr-1, Pax2, Pax5, Wnt1, En1	+	35	Serotonin	Lee et al., 2000
Mouse, transfected with Nurr-1	Five-stage protocol. FGF2 following Shh, FGF8	En1	β-tubulin III, En1, Pax2, Otx2	TH, DA, Nurr-1, DAT, Ptx3, AADC	+	78	Serotonin	Kim et al., 2002
Mouse	Differentiated using a five stage in vitro method		Nestin, MAP2, GFAP	TH	+	33	NT	Nishimura et al., 2003
Mouse, transfected with Bcl-XL	Differentiated using a five-stage in vitro method	Pax2, Pax5, Wnt1	Nestin, β-tubulin III, calbindin, GFAP	En1, Nurr-1, Ptx3, DAT, AADC, TH	+	31	Serotonin, GAD67, GluT	Shim et al., 2004
Mouse	GMEM, KSR, pyruvate, glutamine, nonessential amino acid, ME, coculture with BMSCs (PA-6)		β-tubulin III, NCAM, nestin, synaptophysin	TH, Nurr-1, Ptx3, DA	+	30	GAD, VAChT, Seroton	Kawasaki et al., 2000
Mouse	BMSCs, (PA-6) and late BMP4 exposure or Shh	NCAM	β-tubulin III, NCAM	TH, En2	No	65	NT	Mizuseki et al., 2003
Mouse, adherent monoculture	FGF2, Shh, FGF8	Sox 1	β-tubulin III, nestin, GFAP, CNPase	TH	No	NT	GABA	Ying et al., 2003
Mouse, nuclear transfer	FGF2, Shh, FGF8, ascorbic acid		β-tubulin III	TH	+	>50	Serotonin	Wakayama et al., 2001

Mouse, or nuclear transfer	5 d: co-culture on stromal cell line 2 d: SHH, FGF8, SRM 4 d: N2, SHH, FGF8 bFGF. 3 d: ascorbic acid, BDNF	Nestin, β-tubulin III, MAP2	Nestin, β-tubulin III	TH, DAT, Ptx3, Nurr-1, Lmx1 β, Enl	+	50	Other neurotransmitters in different culture conditions	Barberi et al., 2003
Mouse	IL-1β, GDNF, TGF-β_3, NTN, cAMP		Nestin, synaptosin, GFAP	TH, DAT, D2R, Nurr 1, GFAP, En-1	+	40	GABA, serotonin	Rolletschek et al., 2001
Mouse, transfected with Nurr-1 and GFP	Shh, FGF8, ascorbic acid		β-tubulin III nestin, GalC, GFAP	TH, AADC, DAT, Nurr-1, calretinin, calbindin, Aldh2, Ptx3	+	62	ChAT, Glu, GABA, et al., serotonin	Chung, 2002
Primate ESc								
Human embryoid bodies	RA for 10 d	NF-H	NF-200	DRD1, AADC	No	NT	Serotonin receptor ($5HT_{2A}$, $5HT_{5A}$)	Schuldiner et al., 2001
Human embryoid bodies	EGF, FGF2, PDGF IGF1 (3 d) then NT3, BDNF (14–16 d)		β-tubulin III, nestin, NCAM, MAP2, A2B5, GFAP, synaptophysin	TH	No	3	GABA, Glu glycine	Carpenter et al., 2001
Human spheres	RA for 14–21 d (±PDGF, bFGF, EGF)	Nestin, MBP, GFAP, NSE, NF-M	NCAM, vimentin, nestin, β tubulin III, synaptophysin, NF-L, NF-M, MAP2, synaptophysin, GFAP, 04	TH, Pax6	No	<1	Glutamic acid decarboxylase, GABA, Glu, serotonin,	Reubinoff et al., 2001
Human embryoid bodies	Cultured on ornithine/laminin substrate in a medium consisting of DMEM/F12,		NCAM, nestin, musashi1, β-tubulin III, NF-H, GFAP, O4	TH	No	<1	GABA, Glu	Zhang et al., 2001

(Continued)

Table 2 *Continued*

Population of cells	Induction of differentiation	Gene expression of neuronal lineage	Protein markers of neuronal lineage	Dopaminergic markers (protein/RNA)	HPLC for dopamine	% of tyrosine hydroxylase	Other neurotransmitter (protein/RNA)	Ref.
	N2 supplement cAMP, BDNF							
Human (MB03)	N2, FGF2, TGF-α		GFAP, NF-200, NF-160	TH	+	20	GAD	Park et al., 2004
Cynomolgus monkey	BMSCs (PA-6)		β-tubulin III, NCAM, NeuN,	TH, dopamine, Nurr-1, Lmx1b	+	35	Norepinephrine DβH, GAD, serotonin, ChAT	Kawasaki et al., 2002
Cynomolgus monkey	BMSCs (PA-6) and late BMP4 exposure or Shh		β-tubulin III	TH	No	5	ChAT	Mizuseki et al., 2003
M. fascicularis monkey partheno-genesis	FGF2, Shh, FGF8, ascorbic acid		β-tubulin III	TH	+	25	NT	Cibelli et al., 2002
Human embryonal carcinoma cell line NT2	RA for 5 wk then LiCl or FGF1+TEPA+ DA+IBMX+ forskolin		β-tubulin III, tau, GAP43,	TH, DAT, D2R, Nurr-1, Aldh2	+	Up to 75	NT	Iacovitti and Stull 1997; Zigov et al., 1999, 2000; Iacovitti et al., 2001; Stull and Iacovitti, 2001; Sodja et al., 2002; Misiuta et al., 2003
Bone marrow stromal cells (BMSCs)								
Rat	BHA, forskolin, DMSO, heparin K252a, KCl, valporic acid, bFGF, PDGF	NF-M, tau, synapto-physin	β-tubulin III, synapto-physin, tau	TH	No	Few	ChAT	Woodbury et al., 2002

Rat	Vectors construct consisting of the TH gene and GC gene separated by an IRES	NT	NT	TH, GC, L-dopa	L-dopa	Most of the cells into the isolated clone	NT	Schwarz et al., 2001
Human	EGF, bFGF, cAMP, and several growth factors	NEGF2, NSE, glipican 4, necdin, NF-H, NF-M, CD90, nestin	β-tubulin III, NSE, NF-H, nestin, NeuN	TH, AADC, D2DR, VMAT2, α-synuclein, Nurr-1, Pitx3, Aldh1, En1	L-dopa, DOPAC	60	Serotonin	Levy et al., 2003a
Multipotent adult progenitor cells (MAPCs)								
Rat and mouse	Sequentially with bFGF, FGF8, BDNF for 7 d	Otx1, Otx2, Pax2, Pax5, nestin	GFAP, GalC; NF-H; tau, MAP2	AADC, TH	No	30	GABA, serotonin	Jiang et al., 2002a
Mouse	Sequentially with bFGF, FGF8, Shh, BDNF for 7 d, coculturing with astrocytes	Sox1, Otx1, Otx2, Pax2, Pax5, En1, nestin, GFAP, MBP, GABA	Nestin, NF-H, MBP, GFAP, tau	AADC, TH, DAT, DBH, DA, Nurr-1	No	23 (from neuronal markers)	GABA, TPH	Jiang et al., 2003
Adult neuronal stem cells (NSCs)								
Adult rat hippo-campal	Sequentially with FGF2, RA FBS+NGF or BDNF or NT3	TrkA, TrkB, TrkC	MAP2, β-tubulin III, calbindin, GFAP, GalC	TH	No	1	GABA, AChE	Takahashi et al., 1999
Adult rat hippo-campal	Vectors construct consisting of *Nurr-1, Pitx3, Shh-N.* Sequentially with FGF2, RA or forskolin, or FGF8	NT	MAP2	TH, AADC	No	1.5	NT	Sakurada et al., 1999
Adult mouse subependyma of lateral ventricle of forebrain	FGF2, glial cell-conditioned medium	NT	NT	TH	No	0.23	NT	Daadi and Weiss, 1999

(Continued)

Table 2 *Continued*

Population of cells	Induction of differentiation	Gene expression of neuronal lineage	Protein markers of neuronal lineage	Dopaminergic markers (protein/RNA)	HPLC for dopamine	% of tyrosine hydroxylase	Other neurotransmitter (protein/RNA)	Ref.
Adult rat SN progenitor cells	Sequentially DMEM/F12+N2 supplement+ FGF8 or FGF2, DMEM/F12 +FBS+RA for 7 d	NT	Nestin, A2B5, NG2, GFAP, RIP, β-tubulin III	NT	No	NT	NT	Lie et al., 2002

AChE, cetylcholine esterase; Aldh2, Aldehyde dehydrogenase; AADC, 2 L-amino acid decarboxylase; bFGF, FGF2, basic fibroblast growth factor; BDNF, brain-derived neurotrophic factor; BHA, butylated hydroxyanisole; ChAT, choline acetyltransferase; NG2, chondroitin sulfate proteoglycan; CNPase, marker of oligodendrocytes; DOPAC, dihydroxyphenylacetic acid; L-dopa, levodopa; DMSO, dimethylsulfoxide; DA, dopamine; D βH, dopamine -hydroxylase; D2R, dopamine receptor 2; DAT, dopamine transporter; En1, Engrailed 1; En2, Engrailed 2; FBS, fetal bovine serum; FCS, fetal calf serum; GABA, γ-aminobutyric acid; GalC, galactocerebroside; GMEM, Glasgow minimum essential medium; GFAP, glial acidic fibrillary protein; GDNF, glial cell line-derived neurotrophic factor; Glu, glutamate; GAD, glutamate decarboxylase; GluT, glutamate transporter; GFP, green fluorescence protein; GAP43, growth-associated protein; HPLC, high-performance liquid chromatography; IGF1, insulin-like growth factor-1; IL-1β, interleukin-1; IBMX, isobutyl-methylxanthine; IRES, internal ribosome entry site; KSR, knockout serum replacement; Lmx1b, LIM homeobox transcription factor 1b; LiCl, lithium chloride; MAP2, microtubule-associated protein 2; ME, 2-mercaptoethanol; MBP, myelin basic protein; NCAM, neural cell adhesion molecule; NEGF2, neurite growth-promoting factor 2; NF-H, neurofilament heavy; NF-M, neurofilament medium; NGF, nerve growth factor; NSE, neuron-specific enolase; NeuN, neuronal nuclei; NT3, neurotrophin-3; NTN, neurturin; Nurr-1, nuclear receptor-related 1; TPA, phorbol 12-myristate 13-acetate; PDGF, platelet-derived growth factor; RIP, receptor interacting protein; RA, all-*trans* retinoic acid; SRM, serum replacement medium; Shh, Sonic hedgehog; TGF-α or β_3, transforming growth factor-α or β_3; TH, tyrosine hydroxylase; VAChT, vesicular acetylcholine transporter; NT, not tested. NF-L, neurofilament light.

dendrocytes, and mature neurons. The differentiated cells expressed markers of mature neurons such as NF medium (NF-M), MAP2, and synaptophysin. Furthermore, the cultures contained cell-synthesized glutamate, expressed glutamic acid decarboxylase (GAD), GABA and serotonin, and expressed TH. Cells producing TH and serotonin were relatively rare (<1%). Four studies (Schuldiner et al., 2000, 2001; Carpenter et al., 2001; Reubinoff et al., 2001) demonstrated that human ES cells differentiated to mature neuron-like cells expressing mRNA and proteins, TH and AADC, which were necessary for dopaminergic neuron-like production of DA.

In addition, Zhang et al. (2001) described in vitro differentiation, enrichment, and transplantation of neural precursor cells from human ES cells. Upon aggregation to embryoid bodies, differentiating ES cells formed large numbers of neural tube-like structures in the presence of FGF2. Neural precursors within these formations were isolated by selective enzymatic digestion and further purified on the basis of differential adhesion. Following withdrawal of FGF2, they differentiated into neurons, astrocytes, and oligodendrocytes. A small number of neurons (approx 1%) were found to express TH. After transplantation into the neonatal mouse brain, human ES cell-derived neural precursors were incorporated into a variety of brain regions, where they differentiated into both neurons and astrocytes. No teratoma formation was observed in transplant recipients. These studies could serve as a platform for further manipulations with growth and differentiating factors that might eventually enable the derivation of specific dopaminergic neural cells from human ES cells.

Stromal feeder (SDIA-based) protocols were tested in nonhuman primate ES cell lines. Unlike the EB or neural default protocols mentioned above, SDIA also induced primate ES cells to differentiate into TH^+ neurons (Mizuseki et al., 2003). In addition, Kawasaki et al. (2002) also reported that SDIA induces efficient neural differentiation in ES cells derived from *Macaca fascicularis* (cynomolgus monkey), which is commonly used in preclinical studies. Monkey ES cells were cultured on PA-6 cells for 2 wk, and extensive neurites were found in the majority of primate ES cell colonies (97%); at the cellular level, 45% of SDIA-treated primate cells were NCAM-positive neural cells, and 25% were β-tubulin III-positive postmitotic neurons. However, when primate ES cells were cultured on a gelatin-coated dish without PA-6, <1% of cells became NCAM positive. Furthermore, after 2 wk of SDIA induction, 35% of β-tubulin III postmitotic neurons were TH^+. The SDIA-treated ES cells released DA in response to high K^+ depolarizing stimuli, indicating that they contain a significant number of functional dopaminergic neurons. In addition to DA, norepinephrine was also detected. Consistently, 81% of TH^+ neurons (28% of total neurons) were DβH-negative (dopaminergic neurons) on day 18, whereas the rest were DβH-positive (norepinephrinergic/epinephrinergic neurons). Furthermore, RT-PCR analyses showed that SDIA-induced neurons expressed midbrain dopaminergic neuron markers, such as Nurr-1 and Lmx1β, whereas neither undifferentiated ES cells nor feeder cells did. In addition to TH^+ neurons, SDIA-treated primate ES cells gave rise to GAD^+ (GABA-ergic), $ChAT^+$ (cholinergic), and $5HT^+$ (serotonergic) neurons. In comparison, a previous study with mouse ES cells showed that SDIA treatment induces NCAM and β-tubulin III in 92% and 53% of mouse cells, respectively (Kawasaki et al., 2000), indicating that the efficiency of neural differentiation in primate ES cells by SDIA is about half of that in mouse cells. One possible influence on this neural/non-neural ratio might be the qualitative difference of the non-neural cells. Non-neural cells in SDIA-treated primate colonies were mostly fast-growing E-cadherin-negative mesenchymal cells, whereas those in murine colonies developed at slower-growing E-cadherin-positive epithelial cells. Nevertheless, the proportion of transmitter profiles is similar to that found with SDIA-treated mouse ES cells.

Despite considerable in vitro and in vivo data on human ES-derived neural precursors, differentiation into specific neuronal subtypes such as DA, serotonin, or other motor neurons has not yet been reported. Obtaining functional in vivo data using primate ES cells in animal models of PD will be the next major milestone on the road toward future clinical trials with human ES cells.

From Adult Stem Cells to Dopaminergic Neuron-Like Cells

Adult stem cells have been isolated from adult tissues such as brain, bone marrow, skin, and muscle, and they might have a broader developmental potential than originally anticipated (Joshi and Enver, 2002). Compared with ES cells, tissue-specific stem cells have less self-renewal ability; and although they differentiate into multiple lineages, they are not multipotent. In the last 3–4 yr, a number

of studies have shown that tissue-specific stem cells might be able to generate tissue cells from unrelated organs, such as BMSCs, and these cells can also be induced to differentiate to skeletal (Ferrari et al., 1998), cardiac muscle (Makino et al., 1999), and hepatocytes (Petersen et al., 1999), cells that are not part of their normal repertoire. In terms of mechanism, it remains unclear in many cases to what extent plasticity reflects in vitro adaptation, transdifferentiation/cell-type switching, or the small population of multipotent stem cell persistence in adult tissues of stem cells with extensive endogenous or bona fide developmental potentials. These issues must be resolved before the full therapeutic potential of adult-derived stem cells can be realized. However, the fact that stem cells exist in postnatal tissues with previously unknown proliferation and differentiation potential opens up the possibility of using autologous stem cells to treat otherwise incurable degenerative, traumatic, or congenital diseases without immunological consequences. In addition to the obvious immunological advantage of using autologous stem cells, their use is not encumbered by ethical considerations.

ES cells are not the only candidates for stem cell generation. During embryonic development, the pluripotency of ES cells is narrowed to determined stem cells, which give rise to cells of particular tissues (Gage, 1998). Thus, neural stem cells give rise to cells of the nervous system, and hematopoietic stem cells to blood cells. The determined stem cells differentiate into "committed progenitor cells," which retain a limited capacity to replicate and phenotypic fate. In the past decade, researchers have defined such committed stem or progenitor cells from various tissues, including bone marrow, peripheral blood, brain, liver, and cord blood, in both adult animals and humans. Although most cells in adult organs are composed of differentiated cells that express a variety of specific phenotypic genes adapted to each organ's environment, quiescent stem or progenitor cells are maintained locally or in the systemic circulation and are activated by environmental stimuli for physiological and pathological tissue regeneration (Asahara et al., 2000).

The therapeutic potential of adult stem cells appears to be much lower than that of ES cells. First, adult stem cells are difficult to isolate and propagate in culture. In contrast, ES cells are derived rather easily (once an embryo has been obtained), and they grow indefinitely in culture. Second, ES cells can be manipulated genetically by homologous recombination to correct a genetic defect (Rideout et al., 2002). In contrast, adult stem cells can be genetically manipulated only through the introduction of retroviral transgenes, which overexpress genes at variable levels and can lead to insertional mutagenesis and cancer (Check, 2003). Third, ES cells can be coaxed into becoming any type of cell through the use of specific culture conditions or genetic manipulation. The differentiation potential of adult stem cells, however, seems to be restricted (Hochedlinger and Jaenisch, 2003).

On the other hand, the advantage that the adult stem cells have over ES cells is that there is no ethical restriction. Those opposed to using human ES cells suggest the possible use of pluripotent adult stem cells as a way of realizing medical gain without ethical pain. Although researchers generate ES cells from preimplantation embryos in culture, and several countries have sanctioned deriving human ES cell lines from surplus embryos created through in vitro fertilization, some remain uncomfortable with the destruction of human embryos, even those destined never to be implanted in a uterus. Beyond these ethical issues, there are technical obstacles to the use of ES cells. First, these cells can be obtained only from very young embryos, and although several human ES cell lines have been made, they will not be immunologically compatible with most patients who require cell transplants. Thus, researchers will need either to derive many more ES cell lines or to customize ES cells on a patient-by-patient basis by therapeutic cloning. Second, undifferentiated ES cells form teratomas, benign tumors containing a mixture of tissue types, after being transplanted. Thus, ES cells must be reliably differentiated into the appropriate cell type in culture before transplantation (Stuart and Morrison, 2002). In addition, it is easy to maintain adult stem cells in culture in a nondifferentiated state, and they can be used for autologous transplantation without immunological problems. Thus, we will focus on gene and cell therapy using adult stem cells.

Bone Marrow Stromal Cells

The use of cells originating from patients might provide an autologous transplantation strategy that avoids the introduction of foreign material, circumvent many ethical issues, and significantly reduce the need for immunosuppression. In recent years, there has been an increasing interest in adult BMSCs that support hematopoiesis. BMSCs were discovered by Friedenstein (1976;1987) and his associates in the

1970s. They demonstrated that a small fraction of cells from bone marrow adhere to tissue-culture surfaces and that the adherent cells can be differentiated both in culture and in vivo into osteoblasts, chondrocytes, and adipocytes. In addition, these mesenchymal stem cells differentiate into mature connective tissue, muscle, bone, cartilage, and fat cells (Prockop, 1997; Pittenger et al., 1999; Deans and Moseley, 2000). Evidence has accumulated that human, rat, and mouse BMSCs can also be induced to differentiate to neuron-like cells in culture (Sanchez-Ramos et al., 2000; Woodbury et al., 2000, 2002; Black and Woodbury 2001; Deng et al., 2001; Kohyama et al., 2001; Jin et al., 2003; Joannides et al., 2003; Levy et al., 2003a,b; Munoz-Elias et al., 2003; Padovan et al., 2003; Rismanchi et al., 2003; Qian and Saltzman 2004). Following induction, most (up to 80%) stromal cells can exhibit neuronal phenotypes (Woodbury et al., 2000, 2002). Moreover, it was shown that differentiated cells express neuronal protein markers such as NSE, NeuN, NF-M, and TrkA. Recent transplantation studies have suggested that BMSCs transplanted into developing mouse brain can produce limited numbers of astrocytes (Azizi et al., 1998; Kopen et al., 1999). Chopp et al. have reported that transplantation of undifferentiated BMSCs in rats showed therapeutic benefit after traumatic brain injury (Lu et al., 2001a,b, 2002a; Mahmood et al., 2001), ischemic brain injury (Chen et al., 2001a,b; Li et al., 2001b, 2002), or spinal cord injury (Chopp et al., 2000). However, in these postinjury transplantation studies, generally <20% of transplanted cells were immunoreactive for CNS antigens, thereby raising concerns about the lineage of the remaining cells. Other experiments with rodents demonstrated that transplanted bone marrow-derived cells might migrate into various brain regions and develop neuron-like features (Brazelton et al., 2000; Mezey et al., 2000; Hess et al., 2002; Zhao et al., 2002). Furthermore, Mezey et al. (2003a) found Y chromosomes in the human brains of females following transplantation of male bone marrow. Donor cells were found in several selective brain regions, especially in the hippocampus and cerebral cortex. However, other researchers claim that bone-to-brain transdifferentiation might not be a general phenomenon but might reflect fusion with neurons or transient expression of many proteins, including neuronal markers (Holden and Vogel, 2002; Lemischka, 2002; Wurmser and Gage, 2002).

The evidence for differentiation of BMSCs into dopaminergic neuron-like cells is limited. Rat BMSCs were engineered by *trans* genes to express human TH type 2, the rate-limiting enzyme in DA biosynthesis, and GTP cyclohydrolase I (GC), the enzyme providing the tetrahydropterin cofactor for TH (BH_4) (Schwarz et al., 1999). Gene-engineered rat BMSCs synthesized and released L-dopa. When rat BMSCs that synthesized L-dopa were transplanted into the rat model of PD, the L-dopa was converted to DA and behavioral recovery was observed. However, the ameliorative effect of transplanted rat BMSCs was short-lived (up to 7 d), presumably because of inactivation of transgenes introduced into the brain with retroviruses. In the experiments, BMSCs were transduced sequentially with two separate retroviruses, each containing TH or GC driven by the cytomegalovirus promoter (Schwarz et al., 1999). In addition, they have created a 3.4-kb bicistronic construct consisting of the TH and GC genes separated by an internal ribosome entry site (TH-IRES-GC) to avoid the use of two separate retroviruses (Schwarz et al., 2001). They also used a self-inactivating retroviral vector in which a 3′ enhancer sequence in the LTR was deleted, the 5′-LTR was inactivated upon integration into the target cell genome, and the TH-IRES-GC central construct was driven by a promoter of choice. Moreover, small number of rat BMSCs producing L-dopa could continue to express transgenes after a massive expansion in culture by simple low-density plating nearly 3 mo in vitro. However, the BMSCs in these studies did not differentiate into neuron-like cells.

Woodbury et al. (2002) reported a method for inducing rat BMSCs to differentiate into neuron-like cells that express genes associated with neurotransmission. Rat BMSCs maintained in the induction medium for 10 d were significantly heterogeneous in the level of tau expression, which often correlated with the degree of neuronal morphologic differentiation. The specific marker β-Tubulin III, characteristic of mature neurons, was present in virtually all cells. Analysis by RT-PCR indicated that synaptophysin mRNA, which is associated with synaptic vesicles and transmission, was not present in undifferentiated BMSCs but was detected after 24 h of neuronal differentiation and continued to increase thereafter. The synaptophysin protein was detected in cell bodies, as well as varicose, putative transmitter release sites along processes. Moreover, at 10 d of rat BMSCs differentiation, a large population of neuron-like cells expressed ChAT, which catalyzes the synthesis of the excitatory transmitter acetylcholine (ACh). A smaller subpopulation of rat

BMSC-derived neuron-like cells expressed TH. Nevertheless, production of DA or other catecholamine neurotransmitter was not reported.

The therapeutic potential of BMSCs for the treatment of PD was highlighted by a publication from Li et al. (2001a,b). Mouse BMSC prelabeled with bromodeoxyuridine (BrdU), a thymidine analog and marker of newly synthesized DNA, were grafted into the striatum of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD. The grafted MPTP-treated mice exhibited a significant improvement on the rotarod test at 35 d after transplant, compared with nongrafted controls. Immunohistochemistry revealed BrdU-reactive cells in the striatum of grafted MPTP-treated mice at least 4 wk after transplantation. Double staining showed that approx 0.8% of BrdU-reactive cells expressed TH immunoreactivity. Although mouse BMSc injected intrastriatally survive, express TH immunoreactivity, and promote some functional recovery, further investigation is required to understand the mechanism for this recovery. It is not known whether the grafted cells increase production of DA or whether other processes, such as the secretion of neurotrophic factors by marrow-derived cells, mediate the improvement in motor function.

Park et al. (2001) demonstrate that retroviral transduction of mouse marrow cells with the glial cell line-derived neurotrophic factor (GDNF) cDNA, followed by delivery of these engineered cells, results in marrow-derived GDNF-expressing cells within the brain parenchyma. Furthermore, this ex vivo gene-transfer strategy performed 6 wk prior to exposure to the dopaminergic neurotoxin MPTP results in clear protection of nigral neurons and their striatal terminals. The histochemical protection (TH immunoreactive) correlates with behavioral hyperactivity in GDNF mouse BMSC-transplanted mice compared with control BMSC-transplanted animals. The observed behavioral changes are reminiscent of the reported increased locomotion in mice following intrastriatal injection of GDNF (Cheng et al., 1998).

Levy et al., (2003a) demonstrated that human BMSCs (hBMSCs) might change their designation following induction in culture. The differentiation of hBMSCs into neuron-like cells was associated with dramatic morphological changes. Before treatment, hBMSCs displayed a flat, fibroblastic morphology (Fig. 2A), whereas at 24 h of treatment the cells were rounded, exhibited highly retractile cell bodies, and displayed prominent process-like extensions (Fig. 2B). The neuron-like morphology of the cells was retained in up to 26 d of culture (Fig. 2C). The structural changes were accompanied by expression of the tissue-specific neuronal marker NeuN, as indicated by nuclear immunostaining (Fig. 2D). It was also demonstrated, using RT-PCR methods, that the differentiated hBMSCs expressed *Nurr-1*, *Aldh1*, *Pitx3*, and *EN1*, the transcription factors that regulate the midbrain of the DA neuron. Moreover, DA-related genes *AADC, D2 DA receptor (D2DR),* and *DAT* were increased during the differentiation induction (Fig. 3).

Multipotent Adult Progenitor Cells

The population of the multipotent adult progenitor cell (MAPC), a rare cell within human and rat bone marrow mesenchymal stem cultures, can be multiplied >120-fold (Reyes and Verfaillie, 2001; Reyes et al., 2001; Jiang et al., 2002a, 2002b). It was demonstrated that cells capable of differentiating in vitro to cells of the three germ layers could be selected from rodent bone marrow. It was also shown that similar to ES cells, mouse MAPCs injected into the blastocyst contribute to most, if not all, somatic cell lineages including the brain (Keene et al., 2003). Within the brain, region-specific appropriate differentiation occurs (Keene et al., 2003; Zhao et al., 2003a).

Jiang et al. (2002a) described rodent MAPCs, cultured sequentially with bFGF for 7 d, FGF8 for 7 d, and BDNF for 7 d, generated 30% of cells expressing markers of dopaminergic neurons, AADC, and TH immunoreactivity, 20% of serotonin-containing (serotonin-positive) neurons, and 50% of GABA-containing neurons. Neuron-like cells became polarized, as tau and MAP2 were expressed in axonal and somatodendritic compartment, respectively. A particularly useful approach would be if the MAPCs could be administered systemically and find their way to the damaged CNS region, where they could adopt the phenotype of the missing neuron. However, despite the ability of mouse MAPCs to differentiate into neuroectoderm-like cells ex vivo, no significant engraftment of mouse MAPCs was seen in the brain after iv infusion, and rare donor cells found in the brain did not colabel with neuroectodermal markers (Jiang et al., 2002a).

Jiang et al. (2003) recently described that similar to mouse ES cells, mouse MAPCs can also be induced to differentiate in vitro into cells with biochemical, anatomical, and electrophysiological characteristics of midbrain neuronal cells. Mouse MAPCs were cultured sequentially for 7 d with bFGF, FGF8 plus Shh,

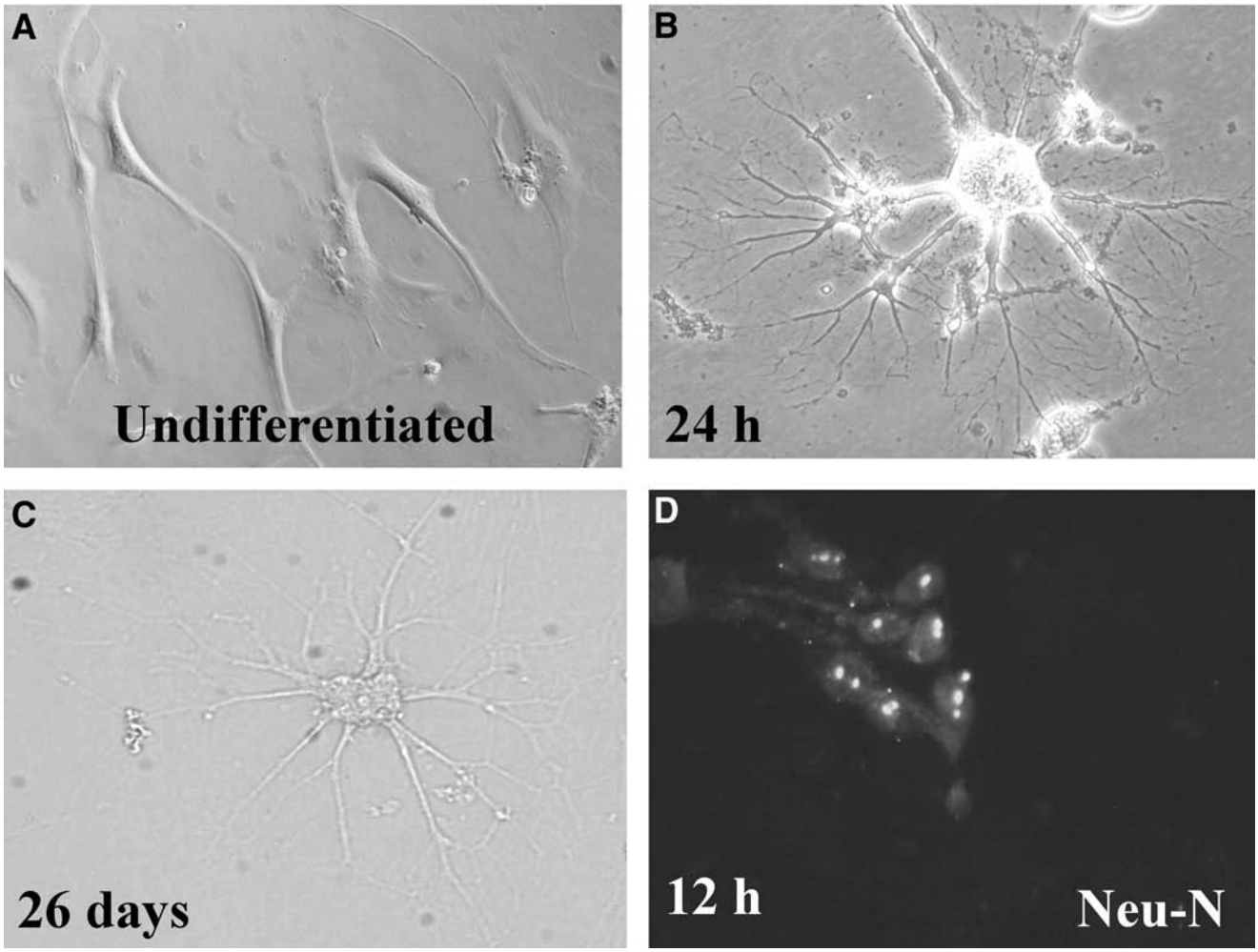

Fig. 2. Formation of neural tissues by hBMSCs. **(A)** Human bone marrow stromal cells (hBMSCs) were isolated from bone aspirate and grown as subconfluent monolayers. **(B)** Cultured hBMSCs were subsequently incubated in a combination of media supplements that induced significant morphological changes. During the first hours of exposure to the induction medium, the cytoplasm in the adherent cells started to condense and retract toward the nucleus, creating a spherical structure; and in some cells, branches around the cells started to develop. **(C)** Differentiated hBMSC display a range of neural-like morphologies after 26 d in culture. **(D)** Immunocytochemistry analysis confirms the expression of NeuN protein after 12 h of differentiation.

BDNF. Immunohistochemistry showed that by day 7 of the differentiation, 23% of nestin-positive cells expressed Nurr-1. Quantitative RT-PCR demonstrated that by days 10 and 14, levels of GABA, TH, and tryptophan hydroxylase (TPH) mRNA increased between 1.7- and 120-fold. Immunophenotypic analysis on day 21 showed that 25% of cells expressed markers of dopaminergic neurons (shown are AADC and TH), 18% expressed markers of serotonergic (TPH) and 52% of GABA-ergic (GABA) neurons. Double immunohistochemistry showed that GABA, TPH, and TH were never detected in the same cells. Mouse MAPC-derived neuron-like cells cultured in the presence of fetal brain astrocytes demonstrated that cells continued to express markers of dopaminergic neurons (25% TH), serotonergic neurons (25% TPH), and GABA-ergic neurons (50% GABA) and acquired a much more mature neural morphology with more elaborate array of axons (Jiang et al., 2003).

Are MAPCs the adult equivalent of ES cells? More data are needed, yet it seems that there are both similarities and important differences between these cells. What, then, are MAPCs? They might be very rare pluripotent stem cells that persist from the embryo into adult life. To prove this it would be necessary to identify these cells prospectively in vivo

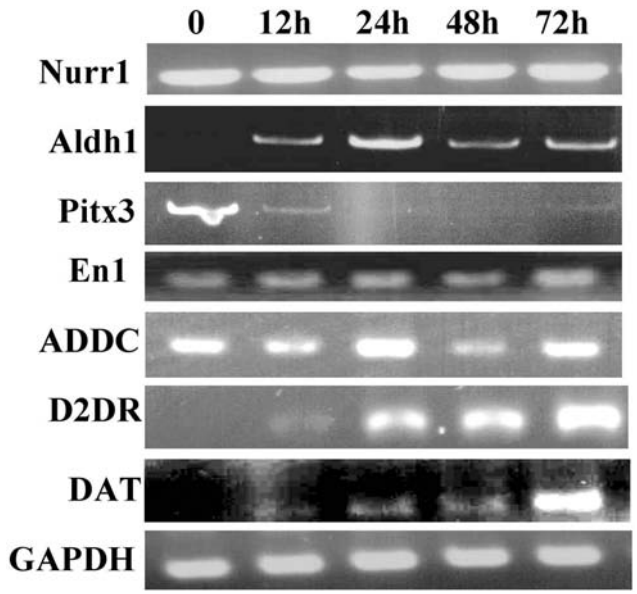

Fig. 3. GAPDH as housekeeping gene. Expression profiles for genes associated with the dopaminergic lineage during hBMSc differentiation. mRNA samples, isolated after 12–72 h of differentiation, expressed markers commonly found in dopaminergic tissues. Aldh1, an aldehyde dehydrogenase capable of metabolizing retinaldehyde into RA; homeobox gene Pitx3; engrailed gene, En1; nuclear receptor related 1, Nurr-1; L-amino acid decarboxylase, AADC; D2 dopamine receptor, D2DR; DA transporter, DAT; and glyceraldehyde 3-phosphate dehydrogenase, GAPDH.

(rather than retrospectively in vitro) by the marker proteins they express and to purify them without an intervening culture step. Alternatively, MAPCs might not actually exist in vivo. The extended period of culture might have triggered certain bone marrow cells to regress to a more primitive state, just as primordial germ cells from which eggs and sperm are produced can be reprogrammed in culture to acquire properties like those of ES cells (Matsui et al., 1992; Donovan 1994). (In fact, ES cells do not exist as such in embryos; they develop after being cultured.) If so, we have much to learn about how cells can be reprogrammed in culture (Stuart and Morrison, 2002).

Multipotent adult progenitor cells (MAPCs) and hBMSCs, irrespective of their origin, might be useful in treating diseases. Until they have been identified in vivo, however, it is too early to speculate their role in repairing injured tissues. Furthermore, although MAPCs and hBMSCs can generate many specialized cell types, it remains to be seen whether those cells function normally and can be used to treat animals (Jiang et al., 2002a). Such experiments will now have high priority, given the possibility of isolating MAPCs and hBMSCs from the bone marrow of any patient and transplanting the progeny of these cells back to the patient without risk of rejection (Stuart and Morrison, 2002). There are great potential benefits in treating diseases by using specialized cells generated in vitro. However, further investigation is needed. There is the risk of ES cells forming teratomas and the unknown hazards of using cells, whether ES cells or MAPCs and hBMSCs, that have been cultured for long periods. In addition, although MAPCs and hBMSCs seem to have normal chromosomes, it is important to establish that the pathways governing cell proliferation are undisturbed. Otherwise, short-range gains might become prey to long-term complications. It underscores the call for research in this area to continue unfettered by political concerns. Only then will the community have a chance to get what it deserves: new, validated, and safe treatments for intractable diseases (Stuart and Morrison, 2002).

Neuronal Stem Cells

The adult vertebrate CNS consists of four major differentiated cell types: neurons, astrocytes, oligodendrocytes, and ependymal cells. Neuronal stem cells (NSCs) are self-renewing multipotent stem cells derived from the CNS with capacity to give rise to cells belonging to all lineages in the CNS such as neurons, oligodendrocytes, and astrocytes (Arenas, 2002). The long-held dogma that we are born with a certain number of nerve cells and that the brain cannot generate new neurons and renew itself has been inverted (Taupin and Gage, 2002). Neurogenesis has been shown to occur throughout adulthood in the adult mammalian brain, and new neurons are generated continuously in some regions of the adult CNS (Corotto et al., 1993; Luskin, 1993). The forebrain subventricular zone (SVZ) and dentate gyrus (DG) are considered to be the major sources of self-renewing, multipotent NSCs (Taupin and Gage, 2002). Neuronal stem cells (NSCs) in the adult SVZ form a cellular continuum with the core of the olfactory bulb (OB) through an extension called the rostral migratory stream (RMS). Cells that originate from the anterior SVZ migrate within the RMS to reside within the OB. Results from in vitro studies with material from human surgical specimens have shown that NSCs can be isolated from regions of the adult human brain, including the wall of the lateral ventricle, cerebral white matter, and the hippocampus (Roy et al., 2000; Palmer et al., 2001). The rate of adult neurogenesis is affected by intrinsic and extrinsic factors. There is no direct evidence for

generation of new neurons in response to acute injury, but the fact that younger patients have better recovery from ischemic stroke than older ones might be partly attributable to a more dynamic stem-cell population existing in younger patients (Armstrong and Barker, 2001). The self-repairing activity of the adult mammal is poor despite the presence of endogenous NSCs. These NSCs can be explained by the microenvironmental factors present in most areas of the adult CNS that might inhibit neuronal differentiation of endogenous NSCs, or by the number of endogenous NSCs that might be too small for effective self-repair (Okano, 2002).

NSCs in Embryonic Brain

Although this review discusses mainly adult and ES cells but not stem cells in the embryo, we will give mention to three different methods that have been used successfully in different groups to induce dopaminergic neurons from NSCs of embryo brain.

Studer et al. (1998) reproduced committed mesencephalic DA neuron precursors from rat embryos in culture. Upon elimination of the mitogen FGF2, part of the cells differentiated into TH^+ assumed dopaminergic neurons. The extended cells survived transplantation to the rat striatum, but the continued existence of the grafted TH^+ cells was poor. Lately, McKay and coworkers reported that the existence of ascorbic acid promotes dopaminergic differentiation when the mesencephalic precursors are proliferated for extended periods in vitro (Yan et al., 2001). Moreover when the predifferentiation of the precursors was carried out in cultures with low oxygen, both proliferation and dopaminergic differentiation were enhanced (Studer et al., 2000). It is not yet known, however, whether ascorbic acid and low oxygen will increase the yield of surviving dopaminergic neurons after transplantation in vivo (Arenas, 2002; Lindvall, 2003).

Wagner et al. (1999) induced a dopaminergic phenotype in an immortalized multipotent mouse NSC line by overexpression of Nurr-1, in a mixture with as yet unidentified factors derived from type-1 astrocytes of ventral mesencephalic source. Most of the Nurr-1-transduced cells expressed the TH enzyme, as well as aldehyde dehydrogenase-2 (Aldh2) and c-ret, two markers of midbrain mesencephalic dopaminergic neurons. The engineered neurons survived transplantation to the mouse striatum, but the yield was very small.

Differentiation of NSCs from the embryonic brain to dopaminergic neurons in vitro and in vivo, as demonstrated above, might offer an effective platform for studying the regulation of cell phenotype. The plasticity of these cells suggests that they can respond to appropriate cues and might be an effective tool to study the progenitor event necessary to generate dopaminergic neurons. However, we do not foresee the use of NSCs from the embryonic brain as cell therapy in PD because of ethical problems and the difficulty of producing these stem cells from the embryo.

NSCs in Adult Brain

During development, neuronal differentiation is influenced by a variety of extracellular signaling molecules that act through nuclear receptors or through one of several cell-surface receptor-mediated signal cascades. The use of RA and forskolin in conjunction with neurotrophic factors such as BDNF or neurotrophin-3 (NT3) has been tested for converting adult hippocampal precursors into dopaminergic neurons. The yield of TH^+ neurons under these conditions remains very modest (<2%), and no evidence of DA neuron function has been reported (Takahashi et al., 1999).

Expression of Nurr-1 in adult NSCs derived from the hippocampus (HC7 or C31 cells), or treatment of these cells with RA or forskolin, was sufficient to induce TH expression (Sakurada et al., 1999). Interestingly, in this study, Nurr-1 was found to bind to the TH promoter and to activate the expression of a GFP reporter, indicating that Nurr-1 promotes transactivation of the TH gene. However, because the induction of TH does not take place in other Nurr-1-expressing NSCs, it seems likely that the induction of TH requires additional factors that would be present in HC7 or C31 cells and that would have to be induced in c17.2 cells. In addition, overexpression of Pitx3 in the same system did not cause an increase in TH^+ cells.

ÅKerud et al. (2001) used c17.2 mouse NSCs engineered to release GDNF, which support the nigral dopaminergic neurons (Lin et al., 1995). The cells grafted well into the host striatum, incorporated and differentiated into neurons (NeuN), astrocytes (GFAP), and oligodendrocytes (CNPase), 1 mo after grafting, and produced high stable levels of GDNF. The percentage of animals showing engraftment at 4 mo reached 12.5% in animals receiving GDNF-c17.2 grafts. However, grafting GDNF-c17.2 cells into adult nude mice resulted in the engraftment of cells in 100% of the animals after 4 mo. Double TH and GDNF immunohistochemistry clearly showed that GDNF

immunoreactivity was contained both in the SN neurophil and within dopaminergic neurons, suggesting that GDNF was efficiently transported in a retrograde manner by dopaminergic neurons from the striatum to the SN. Glial-derived neurotrophic factor (GDNF)-expressing NSCs decreased the loss of SN dopaminergic neurons, increased levels of TH immunoreactivity in the striatum in a 6-OHDA mice model of PD, and showed improvement in behavioral tests.

Yang et al. (2002) have shown that undifferentiated NSCs from newborn mouse cerebellum, transplanted into the rat intact or into 6-OHDA-lesioned striatum, migrated within the host striatum and expressed markers associated with neuronal (β-tubulin III, NSE, and NeuN) but not glial (GFAP, myelin basic protein [MBP^-], $A2B5^-$) differentiation. In 70% of cases, the vast majority of these cells expressed DA-synthesizing enzymes TH and AADC 2–5 wk postgraft. In contrast, no NSCs stained for DβH, ChAT, GAD, or serotonin. As NSCs were capable of migration and differentiation into TH-expressing cells when grafted directly into a 6-OHDA-lesioned striatum, the response of cells, if placed either at a distance from the site of DA denervation or in the absence of DA depletion, was examined. Thus, in this study, NSCs were implanted in the striatum on the side contralateral to a previous 6-OHDA lesion or into the striatum of an intact (unlesioned) rat. When animals were sacrificed 2–4 wk later, it was observed that the NSCs placed contralaterally, as well as those in the intact brain, behaved identically to those placed ipsilaterally. Consequently, some NSCs remained at the graft site, some migrated extensively, reaching as far as the cerebral cortical interface, and other NSCs settled at scattered sites in between. The adjacent tissue expressed TH even though cells were transplanted into host tissue rich in DA innervation. These results suggest that lesion status is not a determinant and thus DA depletion is not a prerequisite for the expression of TH in NSCs. When motor behavior was compared before and after transplantation, no consistent improvement in motor behavior was noted in the cases examined in which grafts expressed TH at 4 wk after transplantation. Although scores declined in five out of six animals, suggesting that striatal DA might have increased on the grafted side, values did not attain statistical significance, indicating that striatal DA had not yet reached levels adequate to reverse their rotational asymmetry. It was concluded that following transplantation into the intact or 6-OHDA-lesioned rat, the adult brain contains intrinsic cues sufficient to direct the specific expression of dopaminergic traits in immature multipotential NSCs. Apparently, the loss of a particular cell type signals the brain to elicit differentiation factors capable of instructing transplanted stem cells in the appropriate phenotypic choices. Natural cues available in the brain can constitutively direct the integration and differentiation of not only some, but virtually all, transplanted NSCs, resulting in their development into neuronal-like cells expressing DA traits. These preliminary data suggest that the TH expressed in engrafted NSCs might produce some measure of DA, a prospect that could improve as TH-expressing NSCs mature, extend a more extensive neuritic network, and integrate more fully into the host brain (Table 1).

The most active neurogenic regions are the DG of the hippocampus and the OB. It has been estimated that approx 10,000 new neurons are added each day to the adult rat DG (Cameron and McKay, 2001), and the rate of neurogenesis in the OB is likely to be severalfold higher. In addition to the neurogenesis in the OB and DG, low numbers of new neurons have been suggested to be generated in other parts of the hippocampus as well as in the cortex (Gould et al., 1999; Rietze et al., 2000), although the latter remains controversial (Kornack and Rakic, 2001). Moreover, recent studies in an animal model of stroke have demonstrated neurogenesis in several additional regions in response to injury (Arvidsson et al., 2002; Nakatomi et al., 2002). Obviously, such self-repair mechanisms, if they are in operation in the adult SN, are insufficient and need to be more effective.

Lie et al. (2002) have shown that the adult rat SN contains a population of actively dividing progenitor cells, which *in situ* give rise only to new mature glial cells but not to neurons. Three days after ip injection of BrdU, cells undergoing DNA replication had incorporated the BrdU, the number of labeled cells in SN appeared to have increased, and cells were found predominantly in doublets, suggesting that they had divided locally. The presence of locally dividing cells in the adult SN was confirmed by stereotactic injection of a Moloney murine leukemia virus (MoMLV)-based GFP retrovirus into the SN. Moloney murine leukemia virus (MoMLV)-based retroviruses infect only dividing cells. At 36 h after injection, GFP-positive cells were detected in the SN. These cells had small, round cell bodies. To determine the phenotype and the fate of dividing cells, animals were injected daily with BrdU for 10 d and perfused at 1 d and 4 wk after injection. Stereological analysis showed that the number of BrdU-positive cells did

not differ significantly between those times, suggesting that a significant proportion of dividing cells or their progeny was maintained during this period. One-half of the BrdU-positive cells in the SN colabeled with the potential glial progenitor marker, chondroitin sulfate proteoglycan (NG2), 1 d after injection (49%). At 4 wk after injection, the percentage of NG2-labeled, BrdU-positive cells had decreased only slightly (42%), suggesting that many cells remained as glial progenitor cells in the SN. At 1 d after injection but not at 4 wk after injection, a small population of BrdU-positive cells (approx 0.1%) stained nestin, which is expressed by multipotent neural progenitor cells during development (Lin et al., 1995). Few newborn cells (<0.1%) expressed mature glial markers (S100) at the early time point. In contrast, at 4 wk after injection, 14% of BrdU-labeled cells had differentiated into oligodendrocytes (13%) or astrocytes (1.4%). Very few BrdU-positive cells (<0.1%) expressed Ox42 at both time points, indicating that few newborn cells in the SN are pluripotent. However, after removal from the adult SN, these progenitor cells immediately have the potential to differentiate into neurons in vitro. After RA-induced differentiation for 7 d, FGF2- and FGF8-treated cultures contained a significantly higher number of cells that had differentiated into neurons (β-tubulin III), astrocytes (GFAP), and oligodendrocytes (receptor-interacting protein [RIP]). Cultures were also treated with BrdU for 48 h before differentiation. After differentiation, the majority (>85%) of neurons and glia were labeled for BrdU, demonstrating that these cells were generated *de novo* from a dividing cell population. Together, these results demonstrate that SN-derived progenitor cells are able to generate cells from all three neural lineages. Progenitor cells were also grafted into the adult SN. However, in contrast to their hippocampus-grafted counterparts, neither FGF2- nor FGF8-stimulated progenitor cells gave rise to any β-tubulin III, NeuN, or TH^+ neurons. Instead, these investigators found that ~30% of the grafted cells showed a glial progenitor phenotype by expressing the marker NG2. These results highlight the role of the environment in the differentiation of SN-derived progenitor cells into neurons in vivo. Because dividing cells or their progeny from the adult SN can give rise to neurons in vitro and in vivo after transplantation, it was hypothesized that cell death of dopaminergic neurons in the SN can induce *de novo* neurogenesis in this region. Animals were injected with 6-OHDA into the left medial forebrain bundle, which leads to a selective loss of dopaminergic neurons in the SN. Dividing cells were labeled by daily injections of BrdU for 12 consecutive days. All sections were screened for newly generated neurons by costaining BrdU with neuronal markers (NeuN, β-tubulin III) and a dopaminergic marker (TH). None of the newly generated cells colabeled for any of these markers. These findings suggest that dopaminergic cell loss induced by 6-OHDA is not sufficient to induce neurogenesis in the SN. The major challenge will be to identify those signals that can drive the progenitor SN cells down a dopaminergic lineage. These results suggest that progenitor cells reside in the adult SN and can give rise to new neurons when exposed to appropriate environmental signals. The presence of these cells in the SN might be very useful in treating PD for endogenous cell-replacement strategies, in which SN dopaminergic neurons degenerate. Further studies should be directed at describing the microenvironment of the SN as a first step in generating an environment permitting dopaminergic neuron differentiation.

Zhao et al. (2003b) reported a slow turnover of dopaminergic projection neurons in the adult rodent brain and that neurogenesis is increased after a partial injury. They provided data supporting the presence of neurogenesis in the adult SN. Substantia nigra (SN) did not lose TH neurons during aging, although TUNEL apoptotic cells were demonstrated in the same neuronal population under the same physiological conditions, which indirectly pointed to the possibility that new nerve cells were added during adulthood to maintain homeostasis. Markers for proliferation, for example, the nucleotide analog BrdU, were demonstrated in nuclei of cells with nerve cell characteristics, that is, the presence of TH, Hu, NeuN, and synapses on the soma. The presence of immature neurons in and around SN containing nestin, some coexpressing TH, further supported evidence of nigral neurogenesis. The newly generated nigral neurons projected axons to the appropriate terminals in the striatum and were integrated into multisynapse circuits to the cortex. Furthermore, the data of Zhao et al. (2003b) suggest that the newly generated dopaminergic projection neurons derive from stem cells lining the cerebroventricular system in the midbrain, with cells putatively migrating along the ventral midline as they express the immature neuronal marker CRMP-4. Although the number of neurons generated is lower than in the hippocampus or the OB, the estimated turnover

rate implies, provided the rate is constant, that the entire population of dopaminergic SN neurons could be replaced within the life span of the mouse. Moreover, systemic doses of MPTP led to a twofold increase of BrdU incorporation in TH nigral neurons 3 wk after the lesion, indicating that the rate of neurogenesis can be regulated. Labeled neurons in the SN did not display pyknotic nuclei or other signs of apoptosis. When MPTP-lesioned animals were killed immediately after BrdU administration, no BrdU incorporation was found in nigral neurons, arguing against BrdU incorporation attributable to DNA repair or apoptotic DNA cleavage. Unveiling the molecular mechanisms controlling neurogenesis might enable the development of strategies to increase the generation of dopaminergic neurons in the adult brain and offer a potentially convenient way to treat PD.

Human NSCs are the most recent addition to the list of novel cells that have been suggested for use in transplantation. These alternative graft sources appear to avoid host immune responses, and their ready availability and multipotentiality are just a few of their advantages over primary fetal tissues. Levesque and Neuman (2002a,b) reported at two international conferences that adult NSCs harvested from a patient's own tissue can be used as a source of DA neurons to aid in the treatment of PD. The finding that adult human brain contains NSCs has raised the hope that the patient's own NSCs could be used to generate DA neurons for neurodegenerative disease such as PD (Table 1).

Possible Mechanisms for Adult Stem Cell Plasticity Differentiation, Transdifferentiation, or Fusion

By definition, stem cells are able to renew themselves and differentiate into at least one mature cell type. Stem cells are subclassified based on their species of source, tissue of origin, and potential to differentiate into one or more specific types of mature cells. Adult (postnatal) stem cells, although still pluripotent, have been thought to have even more limited differentiation ability and to be organ specific. During the last years, the growing number of reports has challenged the traditional concept that stem cells from adult organism, known as tissue-specific stem cells, are capable of producing mature, tissue-specific cells. However, the most versatile tissue appears to be bone marrow, which contains cells capable of changing into unexpected phenotypes such as skeletal, smooth and cardiac muscle, liver, and neural cells (as described above). Although the experimental findings are novel and intriguing, the idea of marrow cells contributing to brain tissue is an old one. *The Yellow Emperors Book of Chinese Medicine* referred to brain as a "sea of marrow" (Morse, 1938). According to this concept, the vital energy (chi) originates in the gonads, flows through the bone marrow, and empties into the sea of marrow, the brain. To the Western scientist, this philosophical view is as strange as the emerging evidence that adult bone marrow cells can metamorphose into brain cells (Song and Sanchez-Ramos, 2003).

Studies on animals and humans document an expanding repertoire of potential for adult stem cells. This flexibility is termed plasticity. An extensively traditional designation of plasticity has yet to be established. Nevertheless, in general, this term refers to the newly discovered ability of adult stem cells to cross lineage barriers and to adopt the expression profiles and functional phenotypes of cells exclusive to other tissues. Many of the results in this new field are controversial, in part, because (1) only a few of the techniques used thus far to assess in vitro and in vivo plasticity are convincing, (2) existing paradigms of cellular differentiation do not yet include postnatal switching of cell fate, and (3) it is unclear how this phenomenon can be safely and reasonably exploited for therapeutic use in humans (Herzog et al., 2003).

What are the proposed mechanisms for adult cell plasticity? Cells have been sorted into unpredicted cellular phenotypes: (1) existence of primitive stem cells in the mature tissue; (2) the presence of multiple progenitor/stem cells not derived from the same embryonic germ layers in the tissue; (3) direct and indirect dedifferentiation; (4) transdifferentiation; and (5) cell fusion. It is of essential importance to keep an open mind about these proposals.

Direct and Indirect Differentiation

Bone marrow cells that differentiate into miscellaneous cell types correspond to an earlier population of extremely pluripotent stem cells placed in the bone marrow and not dedicated to becoming bone marrow derivative cells. In addition, cells might revert to a previous phenotype and be released from their former developmental program. Cells then can redifferentiate in reaction to suitable humoral and cellular signals or they can revert even further to a primordial precursor.

Transdifferentiation

Transdifferentiation refers to the capability of one committed cell type to transform its gene pattern to that of a totally different cell type. Putative mechanisms for this change in potency include indirect transdifferentiation, requiring dedifferentiation followed by maturation down an alternative pathway, and direct transdifferentiation, in which there is a direct transition in the gene expression pattern. Nevertheless, although the difference between direct and indirect transdifferentiation might not be real, existing models of cellular differentiation might not reflect the true plasticity of these cells in vivo (Herzog et al., 2003). Lagasse and others were the first to provide strong support for the concept of transdifferentiation at a functional level by using small numbers of purified stem cells. They showed that as few as 30 highly purified hematopoietic stem cells injected into mice with an inducible lethal hereditary liver disease, tyrosinemia type 1, could repopulate the hematopoietic system, as well as differentiate into hepatocytes and rescue the animals from hepatic failure and death (Lagasse et al., 2000). It is complicated to confirm that the transdifferentiation development does not primarily involve a step back to a more primitive point, particularly if the procedure is fast. If transdifferentiation takes place gradually, then one may be able to identify markers characteristic of earlier stages of development (Song and Sanchez-Ramos, 2003). However, it remains unclear whether the observed plasticity, or transdifferentiation potential, of adult stem cells is inherent in the cells or the consequence of culture conditions, contamination, or cell fusion (Joshi and Enver, 2002).

In an unusual publication of negative data, Shine and colleagues (Castro et al., 2002) described their futile efforts to produce neural cells from bone marrow stem cells. Analysis of 8 mice that received side-population cells from whole bone marrow transplants and 12 that received bone marrow cell transplants revealed no neural-like cells in the CNS. These data suggest that bone to neuron-like cell transdifferentiation might not be a general phenomenon but could depend on the experimental system in which the hypothesis is tested (Castro et al., 2002). However, Mezey et al. (2003b) repeated this work and confirmed that mouse bone marrow cells became neural cells without fusion. In addition, another report has challenged the findings of plasticity: Transdifferentiation of circulating bone marrow hematopoietic stem cells and/or their progeny is an extremely rare event, if it occurs at all (Wagers et al., 2002). This study differs significantly from that of Theise et al. (2003), but the results could not be reproduced. It shows clearly that further experimentation is necessary to determine the factors that inhibit or promote adult cell plasticity response.

Cell Fusion

Another possible mechanism for plasticity could be the fusion of a bone marrow-derived cell with different cell tissue that forms a heterokaryon, thus changing the gene expression shape of the original bone marrow cell type to that of the fusion associate. Somatic cell fusion was first established to take place while cells with mixed karyotypes were identified after the coculture of different cell lines. One of the criticisms of reports on the plasticity of adult stem cells has been that many of the observations can be explained by cell fusion (Terada et al., 2002; Wurmser and Gage, 2002; Ying et al., 2002; Alvarez-Dolado et al., 2003; Medvinsky and Smith, 2003; Prockop et al., 2003; Rudnicki, 2003; Spees et al., 2003; Vassilopoulos and Russell, 2003; Vassilopoulos et al., 2003; Wang et al., 2003). The developmental possibility to modify, by fusion, was shown in studies that established the pluripotency of hematopoietic or neural cells fusing with ES cells (Terada et al., 2002; Ying et al., 2002). In one study primary NSCs cocultured with ES cells fused with them and assumed some of their phenotypic properties (Ying et al., 2002). Using a method based on Cre/lox recombination to detect cell fusion events, it was demonstrated that bone marrow-derived cells fuse spontaneously with neural progenitors in vitro (Alvarez-Dolado et al., 2003). The derived-form offspring were tetraploid and hexaploid. Although fusion is a rare event, it opens the possibility that cells fuse without apparent fusogenic motivation. Therefore, investigators in this field should, when possible, test whether fusion is responsible for gene expression patterns (differentiation) of BMSCs to other cell types. When cell–cell fusion is responsible for reprogramming the gene expression pattern of an adult cell, this still represents plasticity, but cells involved need not be stem cells (Herzog et al., 2003).

It is probable that examples of stem cell plasticity are actually attributable to transplanted donor cells fusing with a different tissue type, followed by reprogramming of the donor cell genome. In principle, this progression might change developmental fate by plasticity, in which extracellular signals must reprogram the genome, because the proper tissue-specific transcription factors are already present in

the cell for fusion. Cell fusion has now been shown to be the cause of the well-characterized example of bone marrow cells forming liver (Vassilopoulos et al., 2003; Wang et al., 2003). Furthermore, bone marrow transplantation demonstrated that bone marrow-derived cells fuse in vivo with hepatocytes in liver, Purkinje neurons in the brain, and cardiac muscle in heart, resulting in the formation of multinucleated cells, the first in vivo evidence for cell fusion of bone marrow-derived cells with neurons and cardiomyocytes (Alvarez-Dolado et al., 2003). No evidence of transdifferentiation without fusion was observed in these tissues.

Because the outcomes of many experiments describing examples of putative stem cell plasticity are also reliable with cell fusion, future studies will be necessary to establish which interpretation is correct. Clinical uses of cell fusion will remain in the distant future until safety issues have been addressed, efficacy has been improved, and whatever therapy is envisaged has become more effective and less toxic than alternative gene transfer approaches based on viral or nonviral vectors (Vassilopoulos and Russell, 2003).

Political, Media, and Legislative Processes

It is generally accepted that ES cells offer the greatest promise for developing new medical treatments. In reality, patients treated adult stem cells have demonstrated much greater potential for curing severe diseases. Yet media reports holding so much sway with public opinion emphasize research concerning stem cells. Any new result, even a minor one, with ES cells is blown up in the media as a major breakthrough. But when researchers announce an even greater success using adult or alternative sources of stem cells, there are generally only small notes relegated to the science pages.

There is an obvious political purpose in the media spin, as it has a major influence on the current cloning debate, both in the United States and abroad. There is a powerful lobby, including biotechnology companies and investors, that is striving to open up scientific research to the therapeutic cloning of human embryos, disregarding the moral objections associated. Although adult stem cell research continues, somehow anonymously, but with remarkable success, the public focus is clearly on ES cell research. The current debate in the US Congress on whether to allow cloning of human embryos for research purposes is a reflection of the present debate.

Human stem cell studies have recently emerged as one of the most controversial areas of biomedical research. Not only are these cells derived from aborted fetuses or from the destruction of fertilized embryos, but their availability has also led to renewed concern about the possibility of human cloning. One of the main arguments advanced in favor of stem cell research is its potential to provide new therapies for otherwise intractable neurodegenerative diseases; therefore, neuroscientists should have a great deal to contribute to this debate. Given that strong feelings are involved on all sides from anti-abortion activists to patient advocacy groups, it is important that the political and public discussion should be informed by a careful evaluation of both the potential and the limitations of NSC therapy.

Obviously, the decision of whether to allow stem cell research to proceed is a political one, which will be made independently in each country. On August 9, 2001, on prime-time television, President Bush announced his decision to allow federal funds to be used for research on existing human ES cell lines on the following conditions: (1) The derivation process (which commences with the removal of the inner cell mass from the blastocyst) has already been initiated; and (2) the embryo from which the stem cell line was derived no longer has the possibility of development as a human being. In addition, President Bush established the following criteria that must be met: (1) The stem cells must have been derived from an embryo that was created for reproductive purposes; (2) the embryo is no longer needed for these purposes; (3) informed consent must be obtained for the donation of the embryo; and (4) no financial inducements have been provided for donation of the embryo. To facilitate research using human ES cells, the NIH is creating a Human Embryonic Stem Cell Registry that will list human ES cells that meet the eligibility criteria. Specifically, the laboratories or companies that provide the cells listed in the registry will have submitted a signed assurance to the NIH. Written documentation to verify the statements in the signed assurance must be retained for submission to the NIH by each provider.

What is needed in the legislative process is a clear perspective about the consequences of such decisions. Scientists cannot achieve progress by overriding basic human values. In this instance, it means developing treatments for various diseases by killing human embryos. One must understand that there is no qualitative difference between cloning a human embryo for therapeutic means (to obtain ES cells)

and for reproductive means (to produce a complete human clone), the difference being only technical. Those who strongly favor therapeutic cloning should admit this fact.

United States legislators are still undecided. The White House supports a strict cloning ban, and many senators are being lobbied and endorse human embryo cloning for medical research only. In Germany, such attempts remain banned, but the importation of stem-cell lines produced by this method from other countries has now been permitted. In Britain, the government has taken a more cautious approach, deciding very recently to postpone any decision on whether the law should be changed to permit therapeutic cloning, pending further examination of the possible benefits and risks. Clearly, the ethical qualms many people feel about interfering with human embryology will have to be weighed against the potential payoff, not only for the neurosciences but also in many other areas. If neuroscientists are to participate effectively in this debate, however, it will be important to represent the field accurately, neither exaggerating the modest results that have been achieved so far nor underselling the enormous potential that might lie ahead. Most importantly, they must make it clear how little we know and how much needs to be done before transplantation therapy becomes a routine option for the treatment of neurological disease.

Future Strategies

In establishing stem cells as an alternative graft source, logistic, ethical, and political issues need to be resolved. There is disagreement over the feasibility of adult stem cells compared with ES cells. Adult stem cells might be capable of developing only into a limited number of cell types, whereas ES cells can form any fully differentiated cell of the body and exhibit remarkable long-term proliferative potential, providing the possibility for unlimited expansion in culture. However, ES cells retain their mitotic ability after transplantation, and this could give rise to tumors. Accordingly, the limited plasticity of adult stem cells might be advantageous in terms of controlling their mitotic ability after transplantation. Furthermore, adult stem cells will not be subject to the ethical concerns that surround the use of fetal tissues, including ES cells (Borlongan and Sanberg, 2002). Thus, safety and efficacy issues on the use of stem cells include the following questions: Do they maintain long-term stable neuronal phenotypes crucial for rescuing the degenerating brain? Are transplanted stem cells functional as a dopaminergic neuron and thus able to provide beneficial effects?

It seems clear that there is an urgent need for more basic research if the field is to progress beyond the level of clinical phenomenology. There are three main challenges: First, it will be necessary to learn much more about neuronal development to define cell types that can be cultured in sufficient quantities and that can adopt appropriate fates when transplanted to different sites in vivo. Second, it will be necessary to establish better animal models perhaps including genetically modified primates to perform more realistic tests of functional and cognitive recovery after transplantation. Third, it will be important to develop methods for testing whether transplanted neurons can become functionally integrated into brain circuitry, that is, whether they can actually contribute to the restoration of normal information processing in the damaged brain. It will require the identification and electrophysiological characterization of transplanted neurons in vivo.

The examples we have provided in this review serve to demonstrate the numerous issues in cell biology to advance a functionally valuable therapeutic strategy in PD. However, the most important scientific conclusion of all the studies with differentiation and transplantation of rodent, primate, and human stem cells is that cell replacement in neurodegenerative disease, in general, and PD, in particular, can work. However, it is essential to emphasize that a clinically helpful cell treatment for PD is not yet available. Dopaminergic cells that are also neurons dopaminergic neurons generated from different sorts of stem cells, seem to be the most hopeful option for grafting in PD. Currently, the best stem cell source for generating new DA neurons is not yet known. However, we believe that adult stem cells such as hMSCs are the best cells to use in autologous transplantation in the treatment of PD.

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