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## Stem Cells as a Source for Cell Replacement in Parkinson's Disease

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Parkinson's disease (PD) is a progressive neurodegenerative disease of the basal ganglia (BG), consisting of a remarkable diversity of neuroactive substances, organized into functional subsystems. Pathologically, it is characterized by continuous dopaminergic cell loss in the nigrostriatal and other dopaminergic systems that are found outside the extrapyramidal system, and its main classic triad of signs involves resting tremor, rigidity, and bradykinesia. The disease affects about 1% of the population more than 50 years of age. Current treatment regimes for PD consist primarily of pharmacologic supplementation of the dopaminergic loss with dopamine (DA) agonist and L-3-4-dihydroxyphenylalanine (L-DOPA, levodopa), a precursor of DA. Levodopa that can readily cross the blood-brain barrier is the most effective agent controlling the symptoms of PD. Most PD 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 treating neurodegenerative disorders such as PD. Given that most neurodegenerative diseases affect the neuronal populations of specific neurochemical phenotypes, an ideal source material for transplantation would be a reproducible cell that could be instructed to assume the desired neuronal phenotype upon differen-

tiation. The strategy of cell replacement therapy seeks to replace the loss in synaptic signaling caused by neuronal degeneration. In late 1970s, 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.<sup>1,2</sup> Encouraged by these findings in animal models, Lindvall and Hagell<sup>3</sup> 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.<sup>3</sup> These clinical tests showed that grafts of fetal ventral mesencephalon successfully survive and reduce motor symptoms.<sup>4–10</sup> Although transplantation is a promising treatment for PD, it requires as many as 5–10 fetal brains for only one PD patient, thus causing ethical and practical problems and 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 has been raised, as a control study pointed to the high risk of adverse signs such as tardive dyskinesia.<sup>11–13</sup> 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 (BMSc) for experimental therapeutics in PD animal models. However, the complete and coordinated induction of specific neuronal phenotype in multipotent neural precursors *in vitro* has proved elusive.<sup>14,15</sup> 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.<sup>16</sup> 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 pharmacologic administration by providing a relatively constant source. Much of the scientific efforts during the past 15 years have therefore had to provide proof-of-principle that (i) the grafted DA neurons can survive and form connections in the PD patient's brain; (ii) the patient's brain can integrate and use the grafted neurons; and (iii) the grafts induce a measurable clinical improvement.<sup>17</sup> The aim of this chapter 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.

## Role of DA in PD

### DA Biosynthesis

Dopaminergic neurons can be identified by the expression of proteins required for the biosynthesis, transport, synaptic packaging, release, and reuptake of DA. DA is produced from the amino acid tyrosine in two steps: TH

catalyses the conversion of tyrosine to L-DOPA followed by decarboxylation to DA via the aromatic L-amino acid decarboxylase (AADC). The striatum contains a dense arborization of the fine terminals derived from the DA-containing neurons of the substantia nigra pars compacta (SNpc). Because these structures contain TH, the DA can be synthesized directly at the terminal varicosities. TH requires iron and tetrahydropteridine in order to oxidize tyrosine to L-DOPA. Only small amounts of L-DOPA are found in the tissue; however, it is readily decarboxylated by AADC. This enzyme is present in many tissues, including serotonergic neurons, where it decarboxylates 5-hydroxytryptophan to form serotonin. Similar to other amino acid decarboxylases, AADC requires pyridoxal phosphate as its coenzyme. DA synthesis in the nerve terminals is accelerated during depolarization-induced release of the neurotransmitter. <sup>[SVM1]</sup>

Two types of transporters are essential to DA neurotransmission: the plasma membrane DA transporter (DAT)<sup>18</sup> and the vesicular monoamine transporter 2 (VMAT2).<sup>19</sup> VMAT2 loads cytoplasmic DA, as well as all other monoaminergic neurotransmitters from the presynaptic nerve terminal into vesicles for storage and subsequent release. DA is rapidly taken up into storage vesicles by an energy-dependent transporter-mediated process. It accumulates extremely high concentrations within the storage vesicle by complexing with adenosine triphosphate and vesicular proteins. When the nerve terminal is depolarized by the arrival of a potential action, calcium enters the nerve terminal through voltage-dependent calcium channels. Local increases in calcium promote vesicle fusion to the nerve membrane and DA is released into the extracellular space.<sup>20</sup> DAT is found exclusively in DA neurons where it terminates the action of DA by rapidly removing it from the synapse.<sup>21,22</sup>

### Specific DA Pathways in the Mammalian Brain

DA is widely distributed throughout the brain, with particularly high concentrations in the striatal areas.<sup>23</sup> Indeed, although DA accounts for about half of the total catecholamines in the brain, more than 80% of brain DA is in the BG.

DA neurons, one of the many types in the brain of higher vertebrates, are located in the mid-brain within the lateral groups of retrorubral field (A8) and the substantia nigra pars compacta (A9), as well as the medially located ventral tegmental area (A10). DA neurons are projected to different forebrain areas, forming the meso-<sup>[SVM2]</sup>encephalic system, where the target neurons are localized in the striatal, limbic, and cortical areas. The substantia nigra pars compacta neurons are connected to the dorsolateral striatum, the caudate putamen, forming the nigrostriatal pathway that is the principal dopaminergic fiber system in the brain and involved in the control of voluntary movement. The neurons of the ventral tegmental area project via the median forebrain bundle to the ventromedial striatum and the subcortical and cortical areas, forming the mesolimbocortical system, which is involved in emotional behavior and mechanisms of natural motivation and reward. Finally, retrorubral field neurons are connected to the substantia nigra and ventral tegmental area and seem to be involved in interconnecting these two areas. They also project to the dorsal striatum via the nigrostriatal pathway.<sup>21,24,25</sup> In addition, there are also DA-containing neurons and terminals in other brain regions, in the retina, and in the spinal cord. The loss of dopaminergic neurons, mainly in the substantia nigra pars compacta and the nigrostriatal pathway seems to have a critical role in Parkinson's and other neurodegenerative diseases.

inverted.<sup>27</sup> 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.<sup>28,29</sup> The forebrain subventricular zone (SVZ) and dentate gyrus are considered to be the major sources of self-renewing, multipotent NSCs.<sup>27</sup> 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 has 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.<sup>30,31</sup> 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 attributed to a more dynamic stem cell population existing in younger patients.<sup>32</sup> The self-repairing activity of the adult mammal is poor despite the presence of endogenous NSCs. This could be explained by the microenvironmental factors present in most of the areas of the adult CNS that may inhibit neuronal differentiation of endogenous NSCs, or by the number of endogenous NSCs that may be too small for effective self-repair.<sup>33</sup>

## Stem Cells in the Central Nervous System

### Neuronal Stem Cells

The adult vertebrate central nervous system (CNS) consists of four major differentiated cell types: neurons, astrocytes, oligodendrocytes, and ependymal cells. Neuronal stem cells (NSCs) are the self-renewing multipotent stem cells derived from the nervous system with a capacity to give rise to cells belonging to all lineages in the nervous system, namely, neurons, oligodendrocytes, and astrocytes.<sup>26</sup> The long-held belief that we are born with a certain number of nerve cells and that the brain cannot generate new neurons and renew itself has been

### Stem Cells in Embryonic Brain

Three different methods have been successfully used to induce dopaminergic neurons from NSCs of embryo brain. Studer et al.<sup>34</sup> reproduced committed mesencephalic DA neuron precursors from rat embryos in culture. Upon elimination of the mitogen basic fibroblast growth factor (bFGF, FGF2), some cells differentiated into TH-positive, assuming dopaminergic neurons. The extended cells survived transplantation to the rat striatum but the survival of the grafted TH-positive cells was poor. Yan et al.<sup>35</sup> reported that the existence of ascorbic acid promotes dopaminergic differentiation when the mesencephalic precursors are proliferated for extended periods *in vitro*. Moreover, when the predifferentiation of the precursors was performed in cultures with

low oxygen, both proliferation and dopaminergic differentiation were enhanced.<sup>36</sup> It is not yet known, however, whether ascorbic acid and low oxygen will increase the yield of surviving dopaminergic neurons after transplantation *in vivo*.<sup>17,26</sup>

Carvey et al.<sup>37</sup> described a method of inducing TH expression by reproducing mesencephalic progenitors from rat embryos with B27 and epidermal growth factor (EGF) in neurosphere cultures. Differentiation was achieved by further treatment with interleukin-11, leukemia inhibitory factor, and glial cell line-derived neurotrophic factor (GDNF). This treatment increased the number of TH<sup>+</sup> cells to 20%–25% of the overall cell population. More recently, the same *in vitro* approach combined with low oxygen has also been used to generate cells expressing dopaminergic markers and releasing DA from human embryonic mesencephalic precursors.<sup>38</sup>

In the third approach, Wagner et al.<sup>39</sup> induced a dopaminergic phenotype in an immortalized multipotent mouse neural stem cell line by overexpressing nuclear receptor related-1 (Nurr1), in a mixture with as yet unidentified factors derived from type 1 astrocytes of a ventral mesencephalic source. Most of the Nurr1 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* (Table 7.1) and *in vivo* (Table 7.2) as demonstrated above, may offer an effective approach for studying the regulation of cell phenotypes. The plasticity of these cells suggests that they can respond to appropriate cues and may 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 complexity 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 retinoic acid and forskolin in conjunction with neurotrophic factors such as brain-derived neurotrophic factor (BDNF) or neurotrophin-3 (NT3) has been tested for converting adult hippocampal precursors into dopaminergic neurons. The yield of TH<sup>+</sup> neurons under these conditions remains very modest (< 2%) and no evidence of dopaminergic neuron function has been reported.<sup>40</sup>

Expression of Nurr1 in adult NSCs derived from the hippocampus (HC7 or C31), or treatment of these cells with retinoic acid or forskolin, was sufficient to induce TH expression.<sup>41</sup> Interestingly, in this study, Nurr1 was found to bind to the TH promoter and to activate the expression of a green fluorescence protein (GFP) reporter, indicating that Nurr1 promotes transactivation of the TH gene. However, because the induction of TH does not take place in other Nurr1-expressing NSCs, it seems likely that TH induction requires that additional factors be present in HC7 or C31 cells, and that they would have to be induced in c17.2 cells. In addition, overexpression of pituitary homeobox 3 (Pitx3) in the same system did not cause an increase in TH<sup>+</sup> cells.

Daadi and Weiss<sup>42</sup> produced a low number of TH-expressing cells from adult mouse forebrain subependyma (SE) *in vitro* by exposure to FGF2 and glial cell conditioned media (CM). They labeled the SE precursor of the adult mouse forebrain *in vivo* by six injections of bromodeoxyuridine (BrdU), a thymidine analog and marker of newly synthesized DNA, given at 2-hour intervals. Thirty minutes after the last BrdU injection, the SE was dissected and cultured in the absence or presence of FGF2 + CM. In control conditions, many cells were labeled with BrdU, but none were TH immunoreactive. However, cultures treated with FGF2 + CM for 1–3 days showed newly generated TH immunoreactivity (0.23% of the total number of cells plated). Of those cells exhibiting TH immunoreactivity, 63% were BrdU-immunoreactive, suggesting that they were derived from the proliferating cells of the adult SE and were born during the 12 hours that preceded the primary culture. Other properties of these cells are unknown.

Akerud et al.<sup>43</sup> used c17.2 mouse NSCs engineered to release GDNF, which support the nigral dopaminergic neurons.<sup>44</sup> The cells

**Table 7.1.** 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	% of TH	Reference
Rodent embryonic stem (ES) cells							
Mouse	Five stage protocol. Stage 4: bFGF, Shh, FGF8; Stage5: AA	nestin, Otx1, Otx2	Nestin, $\beta$ -tubulin III	TH, DA, Nurr1, Pax2, Pax5, Wnt1, En1	+	35	66,90
Mouse transfected with Nurr1	By a five stage protocol. FGF2 after Shh, FGF8	En1	$\beta$ -Tubulin III, En1, Pax2, Otx2	TH, DA, Nurr1, DAT, Ptx3, AADC	+	78	146
Mouse transfected with Bcl-XL	Differentiated using a five-stage in vitro method	Pax2, Pax5, Wnt1	Nestin, $\beta$ -tubulin III, calbindin, GFAP	En1, Nurr1, Ptx3, DAT, AADC, TH	+	31	69
Mouse	SDIA and GMEM, KSR, pyruvate, glutamine, ME, nonessential amino acid		$\beta$ -Tubulin III, NCAM, nestin, synaptophysin	TH, Nurr1, Ptx3, DA	+	30	71
Mouse	SDIA and late BMP4 exposure or Shh	NCAM	$\beta$ -Tubulin III, NCAM	TH, En2	No	65	77
Mouse nuclear transfer	FGF2, Shh, FGF8, AA		$\beta$ -Tubulin III	TH	+	>50	89
Mouse nuclear transfer	5 d: SDIA 2 d: SHH, FGF8, SRM 4 d: N2, SHH, FGF8 bFGF 3 d: ascorbic acid, BDNF	Nestin, $\beta$ -tubulin III, MAP2	Nestin, $\beta$ -tubulin III	TH, DAT, Ptx3, Nurr1, Lmx1b, En1	+	50	75
Mouse	IL-1 $\beta$ , GDNF, TGF- $\beta_3$ , NTN, cAMP		Nestin, synaptophysin, GFAP	TH, DAT, D2R, Nurr1, En-1	+	40	76
Mouse transfected with Nurr1 and GFP	Shh, FGF8, AA		$\beta$ -Tubulin III, nestin, GalC, GFAP	TH, AADC, DAT, Nurr1, calretinin, calbindin, Aldh2, Ptx3	+	62	147
Primate ES cells							
Human embryoid bodies	RA for 10 d	NF-L	NF-H	DRD1, AADC	No	NT	83
Human embryoid bodies	EGF, FGF2, PDGF, IGF1 (3 d) then NT3, BDNF (14–16 d)		$\beta$ -Tubulin III, nestin, NCAM, MAP2, A2B5, GFAP, synaptophysin	TH	No	3	78
Human ES spheres	RA for 14–21 d ( $\pm$ PDGF, bFGF, EGF)	Nestin, MBP, GFAP, NSE, NF-M	NCAM, vimentin, nestin, $\beta$ -tubulin III, synaptophysin, NF-L, NF-M, MAP2, synaptophysin, GFAP, O4	TH, Pax6	No	<1	79

<b>Table 7.1.</b> In Vitro differentiation of stem cells to dopaminergic neural lineages—Cont'd							
Population of cells	Induction of differentiation	Gene expression of neuronal lineage	Protein markers of neuronal lineage	Dopaminergic markers (Protein/RNA)	HPLC for DA	% of TH	Reference
Human embryoid bodies	Cultured on ornithine/laminin substrate in a medium consisting of DMEM/F12, N2 supplement, cAMP, BDNF		NCAM, nestin, musashi1, $\beta$ -tubulin III, NF-H, GFAP, O4	TH	No	<1	82
Human cell line (MB03)	N2, FGF2, TGF- $\alpha$		GFAP, NF-200, NF-M	TH	+	20	84
Human cell line (BG01)	SDIA + GDNF or SDIA + astrocytes originating from either embryonic striatum or embryonic rat ventral mesencephalon			TH, DAT, En1, Pitx3	No	934 cells per well	85
Human cell line (H1, H9, HES-3)	sequential application of SDIA, Shh, FGF8, BDNF, GDNF, TGF- $\beta_3$ , cAMP, AA	MAP2	Nestin, $\beta$ -tubulin III	TH, VMAT2, AADC, En1, Lmx1b, Nurr1, Nurr1, Aldh1	+	79	86
Cynomolgus monkey	SDIA		$\beta$ -Tubulin III, NCAM, NeuN	TH, DA, Nurr1, Lmx1b	+	35	72
Cynomolgus monkey	SDIA and late BMP4 exposure or Shh		$\beta$ -Tubulin III	TH	No	5	77
<i>Macaca fascicularis</i> monkey parthenogenesis	FGF2, Shh, FGF8, AA		$\beta$ -Tubulin III	TH	+	25	92
Human embryonal carcinoma cell line NT2	RA for 5 wk then LiCl or FGF1 + TEPA + DA + IBMX + forskolin		$\beta$ -Tubulin III, tau, GAP43	TH, DAT, D2R, Nurr1, Aldh2	+	up to 75	148–154
Bone marrow stromal cells (BMSc) Rat	BHA, forskolin, DMSO, heparin, K252a, KCl, valproic acid, bFGF, PDGF	NF-M, tau, synaptophysin	$\beta$ -Tubulin III, synaptophysin, tau	TH	No	few	102
Rat	Vectors construct consisting of the TH gene and GC gene separated by an internal ribosome entry site	NT	NT	TH, GC, L-DOPA	L-DOPA	most of the cells	118
Rat/human transfected with NICD	bFGF, CNTF, forskolin (7 d) BDNF + NGF or GDNF (11 d)		Nestin, MAP2, $\beta$ -tubulin III, NF-M	TH, Nurr1, Lmx1b, Pitx3	+	41	114

Human	EGF + bFGF then BDNF + RA	nestin, NeuroD1, Neurog2, musashi1, MBP, $\beta$ -tubulin III, $\alpha$ -synuclein	MAP2, $\beta$ -tubulin III	TH	+ after BH <sub>4</sub>	11	115
Human	EGF, bFGF, cAMP, and several growth factors	NEGF2, NSE, glipican 4, neccdin, NF-H, NF-M, CD90, nestin	$\beta$ -Tubulin III, NSE, NF-H, nestin, NeuN, $\alpha$ -synuclein	TH, AADC, D2DR, VMAT2, Nurr1, Pitx3, Aldh1, En1	L-DOPA, DA, DOPAC	60	109
Multipotent adult progenitor cells (MAPCs)							
Rat and mouse	Sequentially with bFGF, FGF8, BDNF for 7 d	Otx2, Otx1, Pax2, Pax5, nestin	GFAP, GalC; NF-H;tau, MAP2	AADC, TH	No	30	123
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, Nurr1	No	23	122
Adult neuronal stem cells (NSCs)							
Adult rat hippocampus	Sequentially with FGF2, RA, FBS + NGF or BDNF or NT3	trkA, trkB, trkC,	MAP2, $\beta$ -tubulin III, calbindin, GFAP, Gal-C	TH	No	1	40
Adult rat hippocampus	Vectors construct consisting of Nurr1, Pitx3, Shh-N. Sequentially with FGF2, RA or froskolin or FGF8	NT	MAP2	TH, AADC	No	1.5	41
Adult mouse subependyma of lateral ventricle of forebrain	FGF2, glial cell conditioned medium	NT	NT	TH	No	0.23	42
Adult rat substantia nigra progenitor cells	Sequentially DMEM/F12 + N2 supplement + FGF8 or FGF2, DMEM/F12 + FBS + RA for 7 d	NT	Nestin, A2B5, NG2, GFAP, RIP, $\beta$ -tubulin III	NT	No	NT	52

Acetylcholine esterase (AChE); aldehyde dehydrogenase 1/2 (Aldh1/2); L-amino acid decarboxylase (AADC); ascorbic acid (AA); basic fibroblast growth factor (bFGF, FGF2); brain-derived neurotrophic factor (BDNF); butylated hydroxyanisole (BHA); choline acetyltransferase (ChAT); chondroitin sulfate proteoglycan (NG2); ciliary neurotrophic factor (CNTF); CNPase (marker of oligodendrocytes); dihydroxyphenylacetic acid (DOPAC); 3,4-dihydroxyphenylalanine (L-DOPA); dimethylsulfoxide (DMSO); dopamine (DA); dopamine  $\beta$ -hydroxylase (DBH); dopamine receptor 2 (D2R); dopamine transporter (DAT); Engrailed 1 (En1); Engrailed 2 (En2); fetal bovine serum (FBS); fetal calf serum (FCS);  $\gamma$ -aminobutyric acid (GABA); galactocerebroside (GalC); glasgow minimum essential media (GMEM); glial acidic fibrillary protein (GFAP); glial cell line-derived neurotrophic factor (GDNF); glutamate (Glu); glutamate decarboxylase (GAD); glutamate transporter (GluT); green fluorescence protein (GFP); growth-associated protein (GAP43); high-performance liquid chromatography (HPLC); insulin-like growth factor-1 (IGF1); interleukin-1 $\beta$  (IL-1 $\beta$ ); isobutyl-methylxanthine (IBMX); knockout serum replacement (KSR); LIM homeobox transcription factor 1 beta (Lmx1b); lithium chloride (LiCl); microtubule-associated protein 2 (MAP2); 2-mercaptoethanol (ME); myelin basic protein (MBP); neural cell adhesion molecule (NCAM); neurite growth-promoting factor 2 (NEGF2); neurofilament heavy (NF-H); neurofilament light (NF-L); neurofilament medium (NF-M); nerve growth factor (NGF); neurogenic differentiation 1 (NeuroD1); neurogenin 2 (Neurog 2); neuron specific enolase (NSE); neuronal nuclei (NeuN); neurotrophin-3 (NT3); neurturin (NTN); Noch intracellular domain (NICD); not tested (NT); nuclear receptor related-1 (Nurr1); phorbol 12-myristate 13-acetate (TPA); platelet-derived growth factor (PDGF); receptor interacting protein (RIP); *all-trans* retinoic acid (RA); serum replacement medium (SRM); sonic hedgehog (Shh); stromal cell-derived inducing activity (SDIA); tetrahydrobiopterin (BH<sub>4</sub>); transforming growth factor- $\alpha$  or  $\beta$ <sub>3</sub> (TGF- $\alpha$  or  $\beta$ <sub>3</sub>); tyrosine hydroxylase (TH); vesicular acetylcholine transporter (VAcHT); unified PD rating scale (UPDRS).

**Table 7.2.** In vivo differentiation of stem cells to neural lineages after transplantation

Population of Cells/Source	Differentiation	Animal model	Dopaminergic and neuronal markers (surviving TH <sup>+</sup> cells)	Behavioral test	Behavioral recovery	Reference
Embryonic stem (ES) cells Mouse	With or without RA	6-OHDA-lesioned nigrostriatal, rat model for PD	TH (14,500), NSE, NF-H	NT	NT	64
Mouse	Small number of undifferentiated cells	Lesioned the median forebrain bundle by 6-OHDA, rat model for PD	TH (2100), NeuN, DAT, AADC, AHD2, calretinin, calbindin, serotonin	Rotation, PET	+	.65
Mouse transfected with Nurr1	By a five-stage protocol	Lesioned striatum by 6-OHDA, rat model for PD	TH (NT), calbindin	Rotation, step, paw-reaching, cylinder	+	146
Mouse	Differentiated using a five-stage in vitro method	6-OHDA-lesioned striatum, mouse model for PD	TH (NT)	Rotational	+	70
Mouse transfected with Bcl-XL	Differentiated using a five-stage in vitro method	Lesioned substantia nigra by 6-OHDA, rat model for PD	TH (18,310)	Rotational, step	+	69
Mouse	ES colonies were cultured on PA6 cells (12 d)	6-OHDA lesioned striatum, mouse model for PD	TH (13,000), $\beta$ -tubulin III	NT	NT	71
Mouse	ES colonies were cultured on PA6 cells (12 d)	6-OHDA lesioned striatum, mouse model for PD	TH, $\beta$ -tubulin III	NT	NT	73
Cynomolgus monkey	ES cells were cultured on PA6 cells for 3 wk	6-OHDA lesioned striatum, mouse model for PD	TH (830)	NT	NT	72
Mouse or nuclear transfer	5 d: coculture on stromal cell line 2 d: SHH, FGF8, SRM 4 d: N2, SHH, FGF8, bFGF 3 d: N2, AA, BDNF	6-OHDA lesioned striatum, mouse model for PD	TH (23,000), DAT, AADC	Rotational	+	75

Human embryonal carcinoma cell line NT2	LiCl pretreated hNT-DA neurons	6-OHDA lesioned nigrostriatal, rat model for PD	TH (few)	Rotational	No	155
Bone marrow stromal cells (BMSc) Mouse	Undifferentiated	MPTP mouse model of PD: cells grafted in striatum 1 wk later	TH (52)	Rotorod	+	121
Mouse	Vectors construct consisting of the GDNF gene	MPTP mouse model of PD; cells grafted intravenously 6 wk before	TH	Locomotor activity	+	119
Rat	Vectors construct consisting of the TH and GC genes	6-OHDA rat model for PD	TH, GC, L-DOPA	Rotation	+	117
Rat/human transfected with NICD	bFGF, CNTF, forskolin (7 d) GDNF (11 d)	6-OHDA rat model for PD	TH (45%), DAT	Rotation, step, paw-reaching	+	114
Neural stem cells (NSCs) c17.2 mouse	NSCs engineered to release GDNF	6-OHDA lesioned striatum, mouse model for PD	Nestin, NeuN, GFAP, CNPase, TH (350)	Rotation	+	43
c17.2 mouse	Undifferentiated	6-OHDA lesioned striatum, rat model for PD	$\beta$ -Tubulin III, NSE, NeuN, TH, AADC	Rotation	No	156
Human	Differentiation medium (not described)	Human PD	Not relevant	UPDRS	+	55,56

engrafted well in the host striatum, incorporated, and differentiated into neurons (neuronal nuclei, NeuN), astrocytes (glial acidic fibrillary protein, GFAP), and oligodendrocytes (CNPase), 1 month after grafting, and produced high stable levels of GDNF. The percentage of animals showing engraftment at 4 months reached 12.5% in animals receiving GDNF-c17.2 grafts. However, grafting GDNF-c17.2 cells in adult nude mice resulted in the engraftment of cells in 100% of the animals after 4 months. Double TH and GDNF immunohistochemistry clearly showed that GDNF immunoreactivity was contained both in the substantia nigra neuropil and within dopaminergic neurons, suggesting that GDNF was efficiently transported in a retrograde manner by dopaminergic neurons from the striatum to the substantia nigra. GDNF-expressing NSCs decreased the loss of substantia nigra dopaminergic neurons, and increased levels of TH immunoreactivity in the striatum in a 6-OHDA mouse model of PD and showed improvement in behavioral tests.

Yang et al.<sup>45</sup> have shown that undifferentiated NSCs from newborn mouse cerebellum, transplanted into the rat intact or 6-OHDA-lesioned striatum, migrated within the host striatum, and expressed markers associated with neuronal [ $\beta$ -tubulin III, NeuN, and neuron specific enolase (NSE)] but not glial [GFAP<sup>-</sup>, myelin basic protein (MBP<sup>-</sup>), A2B5<sup>-</sup>] differentiation. In 70% of cases, the vast majority of these cells expressed the DA-synthesizing enzymes TH and AADC, 2–5 weeks postgraft. In contrast, no NSCs stained for DA  $\beta$ -hydroxylase (DBH), choline acetyltransferase, glutamate decarboxylase (GAD), or serotonin. Because 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 weeks later, it was observed that the NSCs placed contralaterally as well as those in the intact brain behaved identically to those placed ipsilaterally. It was concluded that, after 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 may produce some measure of DA, a prospect that could improve as TH-expressing NSCs mature, may extend a more extensive neuritic network, and integrate more fully into the host brain.

The most active neurogenic regions are the dentate gyrus (DG) of the hippocampus and the OB. It has been estimated that approximately 10,000 new neurons are added each day to the adult rat DG,<sup>46</sup> and the rate of neurogenesis in the OB is likely to be several-fold 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,<sup>47,48</sup> although the latter remains controversial.<sup>49</sup> Moreover, recent studies in an animal model of stroke have demonstrated neurogenesis in several additional regions in response to injury.<sup>50,51</sup> Obviously, such self-repair mechanisms, if they are in operation in the adult SNpc, are insufficient and need to be more effective.

The question whether the dopaminergic neurons in the SNpc can be divided locally is still unresolved. Lie et al.<sup>52</sup> have shown that the adult rat SNpc contains a population of actively dividing progenitor cells, which in situ give rise only to new mature glial cells but not to neurons.

Zhao et al.<sup>53</sup> reported a slow turnover of dopaminergic projection neurons in the adult rodent brain, and that neurogenesis is increased after a partial injury. However, using similar methodologic procedures, Frielingsdorf et al.<sup>54</sup> argue that they found no evidence of new dopaminergic neurons in the SNpc, either in normal or 6-OHDA-lesioned hemi-parkinsonian rodents, or even after growth factor treatment. Furthermore, they claim that there is no evidence of NSCs emanating from the cerebroventricular system and migrating to the substantia nigra. They conclude that it is unlikely that dopaminergic neurons are generated in the adult mammalian substantia nigra.

Human NSCs have been suggested for use in transplantation. This alternative graft source seems 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<sup>55,56</sup> discovered that adult NSCs harvested from a patient's own tissue can be used as a source of dopaminergic neurons to aid in the treatment of PD. The researchers' methodology included isolating adult human NSCs, expanding them in vitro, inducing them to differentiate into DA-secreting neurons, and selectively delivering them back to targets within the patients. Two years after the procedure was performed, the patient showed no symptoms of PD. Autologous stem cell transplantation has numerous advantages in the treatment of PD. The approach eliminates immune reactions at the site of implantation and improves the likelihood of survival of surgically implanted cells. It also minimizes risks of transmission of infectious disease, and does not require immunosuppressants or steroids. Finally, it does not involve the controversial use of fetal tissue or embryonic cell lines. A major finding is that adult NSCs harvested from a patient's own tissue can be used as a source of dopaminergic neurons. But the key advantage is that the restorative procedure seems safe and effective, with the PD patient experiencing a regression in motor symptoms. The finding that adult human brain contains NSCs have raised the hope that the patient's own NSCs could be used to generate DA neurons for neurodegenerative diseases such as PD.

## Embryonic Stem Cells as a Source for Dopaminergic Neurons

### Embryonic Stem Cells

Embryonic stem (ES) cells are pluripotent cells isolated from the inner cell mass, a cluster of a few hundred identical cells in the blastocyst, which is formed in the early stage of embryonic development. ES cells were first isolated from mouse embryos more than 20 years ago.<sup>57,58</sup> More recently, monkey ES cells were isolated first from the rhesus monkey and then from the marmoset and cynomolgous.<sup>59-62</sup> Shortly after, the same methods were used to isolate human

ES cells from in vitro fertilized human embryos and their potential clinical applications became evident.<sup>61,63</sup> ES cells can proliferate extensively in an undifferentiated state and can provide an unlimited source of many tissue types. The isolation of ES cells from monkey and human embryos has generated great interest in using these cells as a basis of cell replacement therapies for degenerative diseases, especially in PD.

### Rodent ES Cells

#### *Differentiation of ES Cells to Dopaminergic Neuron In vivo*

To assess the potential of ES cells to undergo neuronal differentiation in vivo, totipotent stem cells from mouse blastocysts were transplanted, with (0.5 mM) or without *all-trans* retinoic acid (RA) pretreatment, into adult mouse brain and adult lesioned rat brain.<sup>64</sup> 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, NSE, TH, serotonin, and cells immunoreactive for GFAP. Although graft size and histology were variable, typical grafts of 5–10 mm<sup>3</sup> contained 10–20,000 TH<sup>+</sup> neurons, whereas DBH<sup>+</sup> cells were rare. Both TH<sup>+</sup> and serotonergic axons from intracerebral grafts grew into regions of the DA-lesioned host striatum. These findings demonstrate that transplantation to the brain can induce a significant fraction of totipotent ES cells to become putative dopaminergic or serotonergic neurons and that, when transplanted to the brain, these neurons are capable of innervating the adult host striatum.

Björklund et al.<sup>65</sup> also demonstrated that undifferentiated mouse ES cells grafted in small numbers into the striatum of a 6-OHDA-lesioned rat survived for 14–16 weeks, and developed into normal midbrain-like DA neurons that expressed dopaminergic and neuronal markers, such as TH, NeuN, DAT, and AADC. All TH-positive 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 dopaminergic

neurons. In addition, the differentiated ES cells developed numerous serotonergic neurons and small amount of  $\gamma$ -aminobutyric acid (GABA) as well as choline acetyltransferase (ChAT) neurons. Numerous astrocytes stained for the astrocyte marker GFAP within the grafts were found. Moreover, the animals with ES cell-derived DA neurons recovered from amphetamine-induced turning behavior, and position emission tomography (PET) imaging of presynaptic markers such [ $^{11}\text{C}$ ]CFT found an increase in binding in the grafted striatum (Table 7.2). These results must be reviewed with caution because of teratoma formation and lack of cell survival seen in some rats.

### *Induction of ES Cells to Dopaminergic Neurons In vitro and Function in an Animal Model of PD*

Although the in vivo experiments demonstrated that dopaminergic-like cells can be developed or differentiate by the environmental factor presence in the transplanted niche, much effort is invested to induce differentiation to dopaminergic cells in cultures. McKay and his group at the National Institutes of Health described a five-step procedure.<sup>66</sup> Clusters of ES cells were separated into single cells, and plated for 4 days in the presence of serum. This treatment generated embryoid bodies, floating ES cell aggregates that contain ectodermal, mesodermal, and endodermal derivatives. The embryoid bodies were then plated on adhesive tissue culture plates for 24 hours in the presence of serum and transferred to serum-free medium for 4–6 days to be select for NSCs. These cells were dissociated and cultured for 6 days on an adhesive substrate in the presence of FGF8, sonic hedgehog (Shh), and bFGF. Even though they are essential for the development of midbrain DA neurons in vivo,<sup>67</sup> exogenously added Shh and FGF8 were not essential for the generation of DA neurons from ES cells and only doubled the yield of ES-derived DA neurons. bFGF was then removed, and ascorbic acid was added for a further 6–15 days. The resulting neurons expressed markers that characterize the embryonic midbrain such as Pax2, Pax5, Wnt1, and Engrailed 1 (En1), as well as the dopaminergic neuronal markers Nurrl and TH. Furthermore, these neurons produced DA and released their DA upon depolarization

with potassium or exposure to the neurotransmitter GABA. Under optimal culture conditions, 72% of the ES cells assumed a neuronal morphology, 34% of the neurons were dopaminergic, and 11% were serotonergic. Serotonin and TH were not coexpressed.

An important step forward to increase the proportion of TH<sup>+</sup> neurons was achieved by introducing the Nurrl gene.<sup>68</sup> When cells were treated with FGF8 and Shh, the yield of these cells increased dramatically. Moreover, depolarization markedly increased the DA released in the cultured Nurrl ES cells. Animals grafted with wild-type ES cells showed a slight recovery in rotation behavior in rats lesioned unilaterally with 6-OHDA whereas the group grafted with Nurrl ES cells showed a marked improvement. Interestingly, no teratomas were observed in animals that had received grafts of Nurrl ES cells. These cells also developed functional synapses and demonstrated electrophysiological properties typical to mesencephalic neurons. Shim et al.<sup>69</sup> generated mouse ES cells that constitutively express Bcl-XL, an antiapoptotic protein of Bcl-2 family. In vitro, Bcl-XL overexpressing ES cells resulted in higher expression of genes relating to midbrain dopaminergic neuron development and increased the number of ES-derived neurons expressing midbrain DA markers (TH<sup>+</sup>, 31%), pronouncing the reversal of behavior in the animal model of PD. 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. Additional questions are of long-term safety and whether this type of ES genetic modification of the ES cells is acceptable in a clinical setting.<sup>17</sup>

Nishimura et al.<sup>70</sup> used a similar five-step procedure to differentiate ES cells, carrying the enhanced GFP gene, in a mouse model of PD induced by 6-OHDA. Immunocytochemical evaluations revealed that microtubule-associated protein 2 (MAP2), TH<sup>+</sup>, GFAP<sup>+</sup>, and nestin<sup>+</sup> cells comprised approximately 80%, 30%, 10%, and 5%, respectively, of the whole cells at stage 5. Furthermore, DA and levodopa were detected in the culture supernatants by high-performance liquid chromatography (HPLC) after a 10-day culture in stage 5. These differentiated ES cell-derived cells were used as allografts in mice after administration of 6-OHDA injections. Four weeks after 6-OHDA injection, mice were transplanted with 10<sup>5</sup> ES-GFP cells into their DA-

[SVM4]

denervated striata. Improved rotational behavior was observed 2 weeks after transplantation. TH<sup>+</sup> cells were found at the grafted sites 8 weeks after transplantation, some of which were immunopositive to GFP, demonstrating the presence of dopaminergic neurons derived from the ES cells.

Kawasaki et al.<sup>71,72</sup> screened for DA neuronal differentiation of ES cells after coculture with various cell lines and discovered that the bone marrow (BM)-derived stromal cell line, PA6, is a potent inducer of neuronal differentiation. This was termed stromal cell-derived inducing activity (SDIA). After coculture with PA6 cells, 16% of the total cell population represents DA neurons. Whereas coculture of ES cells with the PA6 cell line induces neural differentiation, CM from PA6 cells does not. However, SDIA is also not blocked by a 0.4- $\mu$ m membrane barrier, suggesting that the inducing activity is secreted, but may be labile. When transplanted, SDIA-induced dopaminergic neurons integrate into mouse striatum, previously treated with 6-OHDA, and remain positive for TH and  $\beta$ -tubulin III expression. These cells survive at a reasonable rate (20%) 2 weeks after implantation in mouse striatum and extend dopaminergic neurites into the target tissue. No teratoma formation was observed in the grafted cells by histologic analyses. Morizane et al.<sup>73</sup> optimize the transplantation efficiency and found that the ratio of the number of surviving TH-positive cells to the total number of grafted cells was highest when ES cells were treated with SDIA for 12 days before transplantation. The SDIA method has several advantages in producing neurons, compared with previous embryoid body methods. First, the SDIA method is technically simple. ES cells are grown in a flat culture from single cells into colonies. Second, neural differentiation of ES cells is not only efficient but also speedy; the postmitotic neuron marker,  $\beta$ -tubulin III, appears on day 4 or 5. Finally and most importantly, SDIA-treated ES cells differentiate into midbrain dopaminergic neurons at a high frequency: 30% of neurons derived from mouse ES cells are dopaminergic and produce significant amounts of DA.

Ying et al.<sup>74</sup> monitored the production of neurons during monolayer differentiation of mouse ES cells. Undifferentiated ES cells were dissociated and plated onto gelatin-coated tissue culture plastic at a density of  $0.5\text{--}1.5 \times 10^4/\text{cm}^2$  in N2-B27 medium. Many of the neurons formed in

N2-B27 are immunopositive for GABA, with few TH-positive cells. However, replating and the addition of FGF2, FGF8, and Shh resulted in significant numbers of TH-immunoreactive neurons. Therefore, neural precursors generated by monolayer differentiation are malleable and can be directed into particular neuronal fates. They can also produce both astrocytes and oligodendrocytes.

Barberi et al.<sup>75</sup> improved techniques for in vitro differentiation of mouse ES cells into several subtypes by providing a set of coculture conditions that allows rapid and efficient derivation of most CNS phenotypes. The conditions induced neural differentiation by coculturing ES cells with murine BM-derived stromal feeder cell lines. After 5 days of coculture ES cells on stromal cell line, Shh and FGF8 were added to the serum replacement medium, and then the medium was changed to N2 supplemented with Shh and FGF8 in the presence of bFGF (days 8–11). At day 11, terminal differentiation was induced by withdrawal of Shh, FGF8, and bFGF and the addition of ascorbic acid and BDNF. These cells expressed TH, and the midbrain-specific transcription factors En1, Nurr1, Pitx3, and LIM homeobox transcription factor 1 beta (Lmx1b), as well as the DAT. 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, the measurement of neurotransmitter release, and intracellular recording.

Using a different approach with pluripotent mouse ES cells, Rolletschka et al.<sup>76</sup> used an efficient protocol for growth factor-mediated lineage selection of neuronal cells. The protocol includes proliferation and maintenance of neural precursor cells in the presence of bFGF and EGF. Differentiation was induced by withdrawal of bFGF/EGF, and the combined addition of neurobasal medium plus B27, fetal calf serum, and the survival-promoting factors (SPF). Interleukin-1 $\beta$  was added daily and adenosine 3',5'-cyclic monophosphate (cAMP) at every 4 days for 30 days. GDNF and transforming growth factor (TGF)- $\beta_3$  were applied beginning at day 4 and at day 7 of the differentiation, respectively, and neurturin (NTN) was applied on day 7. Four days after plating, 58% of ES cell-derived neural precursor cells were labeled by the nestin and the application of SPF between

days 14 and 30 resulted in a significant increase in the frequency of TH (43%) and DAT (39%) positive cells.

## Primates' ES Cells

### *Nonhuman Primate ES Cells*

Stromal feeder (SDIA-based) protocols were tested in nonhuman primate ES cell lines. Unlike the embryoid bodies or neural default protocols mentioned above, SDIA also induced primate ES cells to differentiate into TH<sup>+</sup> neurons.<sup>77</sup> In addition, Kawasaki et al.<sup>72</sup> also reported that SDIA induces efficient neural differentiation in ES cells derived from *Macaca fascicularis* (cynomolgus monkey), which is frequently used in preclinical studies.

### *Development of Dopaminergic Neurons from Human ES Cells*

**Production of tyrosine hydroxylase-positive neurons.** Since the derivation of human ES cell lines from preimplantation embryos in 1998 by Thomson et al., considerable research is centered on their biology, and how differentiation can be encouraged toward particular cell lineages. Various studies have described the potential of human ES cells to differentiate into multiple lineages such as neural progenitor,<sup>78–80</sup> hematopoietic precursors,<sup>81</sup> and insulin-secreting cells (Assady et al., 2001).

Differentiation protocols in primate ES cells to neuron are based on the concepts developed for mouse ES cell differentiation. Multistage embryoid bodies-based differentiation protocols for human ES cells have been reported achieving efficient derivation of neuronal and astrocytic fates.<sup>78,82</sup> Carpenter et al.<sup>78</sup> described that human ES cells were maintained for more than 6 months in vitro (more than 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 and A2B5. After further maturation, these cells

expressed additional neuron-specific antigens and TH. 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.

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.<sup>63,79</sup> reported the generation of enriched and expandable preparations of proliferating neural progenitors from human ES cells. The neural progenitors could differentiate in vitro into the three neural lineages – astrocytes, oligodendrocytes, and mature neurons. The differentiated cells expressed markers of mature neurons such as neurofilament medium (NF-M), MAP2, and synaptophysin. Furthermore, the cultures contained cells synthesized glutamate, expressed GAD, GABA, and serotonin, and expressed TH. Cells producing TH and serotonin were relatively rare (<1%). These four studies<sup>78–80,83</sup> demonstrated that human ES cells differentiated to mature neuron-like cells expressing mRNA and proteins that were necessary for dopaminergic neuron-like TH, and AADC for production of DA. [SVM5]

In addition, Zhang et al.<sup>82</sup> described the 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. After withdrawal of FGF2, they differentiated into neurons, astrocytes, and oligodendrocytes. A small number of neurons (approximately 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 may serve as a platform for further manipulations with growth and differentiating factors that may eventually enable the derivation of specific dopaminergic neural cells from human ES cells.

**Derivation of midbrain dopaminergic neurons.** 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. For human ES cells to be used for transplantation into patients with PD, they must be differentiated into DA neurons with no residual ES cells. Although protocols<sup>78–80,82,83</sup> have been developed for the directed differentiation of human ES to TH-positive neuron, even up to 20% TH-positive cells reported,<sup>84</sup> but there was no confirmation of midbrain DA neuron identity. The first step through achieving derivation of midbrain DA neurons from human ES cells was demonstrated by Buytaert-Hoefen et al.<sup>85</sup> TH-positive neurons with neuronal morphology were generated from human ES cells, after 3–4 weeks in culture, by coculturing on PA6 stromal cells and addition of GDNF to the differentiated media. Moreover, there was RNA expression of *En1*, *Pitx3*, and *DAT*, several factors involved in the development of DA neurons.

Another experiment was directed toward showing that TH-positive cells produced from human ES cells are authentic midbrain DA neurons.<sup>86</sup> Perrier et al.<sup>86</sup> described that midbrain dopaminergic neuron differentiation of human ES was triggered by four stages: 1. Form ES to rosette neural precursors by stromal feeder cells (MS5 stromal cells) for 28 days; 2–3. committed DA precursors was achieved under sequential application of *Shh*, FGF8, BDNF, and ascorbic acid 7–9 days (two passage); 4. DA neuron differentiation required withdrawal of *Shh*/FGF8 and exposure to BDNF, GDNF, TGF- $\beta_3$ , cAMP, and ascorbic acid for 7–9 days. Progression toward a midbrain DA neuron fate was monitored by the sequential expression of key transcription factors, including *Pax2*, *Pax5*, *Lmx1b*, *Aldh1*, *Nurr1*, and *En1*; 30%–50% of the total cells were  $\beta$ -tubulin III-positive neurons, and among them 64%–79% of the cells expressed TH; measurements of DA release by HPLC; the presence of tetrodotoxin-sensitive action potentials; and the electron microscopic visualization of TH-positive synaptic terminals. One important application of the reported midbrain DA neuron derivation protocol will be transplantation into preclinical animal models of PD.

Khaner et al.<sup>87</sup> have developed protocols that allow differentiation of human ES cell-derived neural progenitors toward enriched populations of dopaminergic neurons. The expression of the

midbrain marker genes *En1* and *En2* and the dopaminergic markers TH and AADC was up-regulated in the neural progenitors at an early passage stage, suggesting that they have acquired the potential to adopt a midbrain fate at an early stage of propagation. Based on these data, the neural progenitors have been treated during a specific time window at an early passage stage with FGF8 together with ascorbic acid. Immunofluorescent analysis revealed that this treatment directed the differentiation of the neural progenitors into cultures enriched for TH<sup>+</sup> neurons (approximately 30% of the total neurons generated). The TH<sup>+</sup> neurons that were generated could secrete DA into the culture medium.

The potential of human ES cells to induce functional recovery in an animal model of Parkinsonism was reported for the first time by Reubinoff et al.<sup>88</sup> They generated highly enriched cultures of neural progenitors from hES cells and grafted the progenitors into the striatum of parkinsonian rats. The grafts survived for at least 12 weeks, the transplanted cells stopped proliferating, and teratomas were not observed. The grafted cells differentiated *in vivo* into DA neurons, although at a low prevalence similar to that observed after spontaneous differentiation *in vitro*. Transplanted rats exhibited significant improvement in rotational behavior, and in stepping and placing nonpharmacologic behavioral tests.

The availability of unlimited numbers of midbrain DA neurons is a first step toward exploring the potential of human ES cells in preclinical models of PD. Further developments for the potential use of human ES cells in the treatment of PD are needed. 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.

## Nuclear Transfer and Parthenogenesis ES Cells

Transplantation of allogenic or xenogenic tissues and cells raises immunologic concerns. The need for immunosuppression in neural transplantation remains a controversial issue given the proposed “immunoprivileged” status of the brain. However, it is well established that xenografted cells undergo rapid rejection in the brain without immunosuppression. Nuclear transfer and parthenogenesis are procedures

that might provide immunocompatible cell sources for transplantation.

### *Dopaminergic Neuron Differentiation of ES Cells Generated from Adult Somatic Cells by Nuclear Transfer*

Functional DA neurons were obtained from mouse ES cells established via nuclear transfer from adult tail tip or cumulus cells as described by Wakayama et al.<sup>89</sup> They derived 35 ES cell lines via nuclear transfer (ntES) from adult mouse somatic cells of inbred, hybrid, and mutant strains. ntES embryoid bodies have been induced to differentiate in vitro to produce dopaminergic neurons as described previously<sup>90</sup> with the following minor modifications. Cells were cultured for a longer period during stage III (CNS selection stage), ranging from 9 to 16 days and the concentrations of FGF2, Shh, FGF8, and ascorbic acid were changed. One ES cell line yielded dopaminergic neurons in excess of 50% of the total cell number. The functional nature of these neurons was confirmed by HPLC determination of DA release. Serotonergic neurons were also detected histochemically, although in smaller numbers, and serotonin release was confirmed by HPLC. In combining ES and nuclear transfer technologies, the feasibility of the first steps required for application of cloning to transplant therapy was demonstrated.<sup>66,89,90</sup>

Barberi et al.<sup>75</sup> demonstrated that mouse ntES cell-derived neurons by multistage differentiation protocols have electrophysiological evidence of synapse. Therefore, cloning and long-term expansion of undifferentiated ntES cells do not interfere with the functionality of their differentiated progeny. The ultrastructural detection of typical large, dense-core vesicles suggests that these are dopaminergic neurons. There has been great interest in developing a renewable cell source for the generation of DA neurons in the experimental treatment of PD. Their study demonstrates the functionality of ntES cell-derived neurons in 6-OHDA-lesioned mice, without the requirement of exogenous Nurrl expression. The transplantation results using ntES cell-derived DA neurons in parkinsonian mice demonstrate the efficacy of therapeutic cloning in an animal model of CNS disease. Earlier work has shown in vivo functionality of bovine DA neurons extracted from

cloned fetuses.<sup>91</sup> However, the use of cloned fetuses as cell donors raises ethical barriers that preclude applications in human disease. Notably, the DA neuron survival rate in vivo was higher in ntES than in ES cell-derived grafts (80% versus 40%, 8 weeks after transplantation). The results show the potential of therapeutic cloning in mouse models of PD. Future therapeutic applications may require extensive work to adapt these protocols to human ES cells.

### *Dopaminergic Neuron Differentiation of Parthenogenesis Stem Cells*

Parthenogenesis is the process by which an egg can develop into an embryo in the absence of sperm. Broad differentiation capabilities of non-human primate (*M. fascicularis*) pluripotent stem cells derived by parthenogenesis have been reported.<sup>92,93</sup> Neural differentiation of Cyno-1 cells (one stable cell line from blastocyte's inner cell mass) was induced with a multistep culture procedure,<sup>66,90</sup> and astrocytes and neurons were obtained. Up to 25% of dopaminergic (TH) neurons could be obtained, as judged by immunocytochemical criteria. Neuronal identity and function were confirmed by HPLC analysis, which showed in vitro release of the neurotransmitters DA and serotonin. The in vitro differentiation of these cells to well-characterized dopaminergic neurons is of particular interest, because of their potential to replace lost neurons in PD. The proposal of human therapeutic cloning describes the generation of autologous ES cells through somatic cell nuclear transfer.<sup>94</sup> This study suggests an alternative to human cloning for therapy. Differentiated cell types derived in vitro by parthenogenesis eliminate the requirement to produce or disaggregate a normal, competent embryo and may circumvent the ethical concerns voiced by some, with a positive impact on the debate in stem cell research. Future studies will have to provide a proof-of-principle application of both nuclear transfer and parthenogenesis in animal models of PD.

### **Generation of DA Neurons from Adult Stem Cells**

Adult stem cells are found in different tissues of the adult organism that remain in an undifferen-

tiated, or nonspecialized, state. Adult stem cells possess the ability to self-renew, and can differentiate into at least one tissue-committed cell type. The main function of stem cells in adult tissue is to regenerate the tissue in which they reside. By a traditional developmental paradigm, adult stem cells are able to differentiate only to the tissue in which they reside. Recent data challenge the committed fate of the adult stem cells and present evidence for their plasticity.

Plasticity of the adult stem cells may offer valuable therapy for a broad spectrum of diseases, especially neurodegenerative diseases and brain injuries. The use of cells originating from the patient's BM, skin, or fat cells may provide an autologous transplantation strategy that obviates the introduction of foreign material, circumvents many ethical issues, and significantly reduces the need for immunosuppression. Herein, we will review the potential application of BM-derived stem cell transplantation in PD, although several researchers suggest using skin, fat cells, or other tissues for clinical use.

### Bone Marrow Stromal Cells

BMSC were described by Friedenstein and his associates in the 1970s.<sup>95,96</sup> They demonstrated that a small fraction of cells from BM adhere to tissue culture surfaces and that the adherent cells can be differentiated both in culture and in vivo into osteoblasts, chondrocytes, and adipocytes. It was shown that these stromal cells, known also as mesenchymal stem cells, further differentiate into mature connective tissue, muscle, bone, cartilage, and fat cells.<sup>97-99</sup>

### *Induction of Dopaminergic Neuron-like Cells from BMSc In Vitro*

A series of studies on human, rat, and mouse BMSc found evidence that these cells can be induced to differentiate to neuron-like cells in cultures.<sup>100-116</sup>

The first and most critical step to get neuron-like cells is to isolate the BM-derived multipotent progenitor cells. One of the methods to isolate multipotent cells with broad differentiation potential is based on the size and adherent capacity of the BMSc.

To increase the efficiency of differentiation of neuron-like cells, raising the cAMP levels by dibutyryl cyclic AMP and isobutylmethylxanthine,<sup>104</sup> or addition of the antioxidant butylated hydroxyanisole and dimethylsulfoxide have been used.<sup>101</sup> Another study has suggested that neuronal cells are isolated after induction with noggin, 5-azacytidine, and the neurotrophic factors such as nerve growth factor (NGF), NT3, and BDNF or the combination of FGF2, EGF, NGF, and retinoic acid.<sup>105</sup>

The evidence for differentiation of BMSc into dopaminergic neuron-like cells is limited. Woodbury et al.<sup>102</sup> reported a method for inducing rat BMSc to differentiate into neuron-like cells that express genes associated with neurotransmission. Rat BMSc maintained in induction medium for 10 days were significantly heterogeneous in the level of tau expression, which often correlated with the degree of neuronal morphologic differentiation.  $\beta$ -Tubulin III, an intermediate filament characteristic of mature neurons, was present in virtually all cells. Analysis by reverse transcriptase-polymerase chain reaction (RT-PCR) indicated that synaptophysin mRNA, which is associated with synaptic vesicles and transmission, was not present in undifferentiated BMSc but was detected after 24 hours 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 days of rat BMSc differentiation, a large population of the neuron-like cells expressed ChAT, which catalyzes the synthesis of the excitatory transmitter acetylcholine. A smaller subpopulation of rat BMSc-derived neuron-like cells expressed TH. Nevertheless, they did not report a production of DA or other catecholamine neurotransmitters.

An additional study by Hermann et al.<sup>115</sup> described the efficient conversion of human BMSc into a neural stem cell-like population. These cells grow in neurosphere-like structures, express high levels of early neuroectodermal markers, such as the proneural genes neurogenic differentiation 1 (NeuroD1), neurogenin 2 (Neurog2), musashi 1, as well as orthodenticle homolog 1 (OTX1) and nestin, and lose the characteristics of mesodermal stromal cells. The authors used growth factors such as EGF and FGF2 and later exposed the cells to BDNF and retinoic acid. Marker mRNA levels of mature

neural cell types (GFAP, MBP,  $\beta$ -tubulin III, and TH) were significantly increased. They obtained 42% of differentiated human BMSc with early neuronal characteristics ( $\beta$ -tubulin III expression) and 6% expressing the marker molecule for mature neurons (MAP2). Moreover,  $11\% \pm 7\%$  of cells expressed TH, and DA was detectable in the media of the differentiated cells, by HPLC, only after supplemented with tetrahydrobiopterin ( $BH_4$ ).

In our laboratory, Levy et al.<sup>109</sup> induced differentiation of mouse BMSc to neuron-like cells, without any gene introduction, and observed the activation of the tissue-specific promoter of NSE. We used transgenic (Tg) mice that carry the antiapoptotic human bcl-2 gene, expressed only in neurons under the NSE promoter. We found that, after induction, the mouse BMSc demonstrate neuronal phenotype and express the neuronal markers and the human Bcl-2. Furthermore, we also demonstrated that human BMSc might change their designation after induction in culture.<sup>110</sup> The differentiation of human BMSc into neuron-like cells was associated with dramatic morphologic changes. Before treatment, human BMSc displayed a flat, fibroblastic morphology, whereas, after 24 hours of treatment, the cells were rounded, exhibited highly retractile cell bodies, and displayed prominent process-like extensions. The neuron-like morphology of the cells was retained up to 26 days of culture. The structural changes were accompanied by the expression of the tissue-specific neuronal marker, NeuN, as indicated by nuclear immunostaining. We also demonstrated, using RT-PCR methods, that the differentiated human BMSc expressed *Nurr1*, *Aldh1*, *Pitx3*, and *EN1*, the transcription factors that regulate the midbrain of the DA neuron. Moreover, the DA-related genes *AADC*, *D2* DA receptor, and *DAT* were increased during the differentiation induction.<sup>110</sup>

### *Gene Manipulations and Dopaminergic-like Cells and Functional Assay in an Animal Model of PD*

Because the induction of differentiation BMSc into dopaminergic neuron-like cells is limited, gene manipulations were performed to increase the efficiency and quality of the dopaminergic-like cells. Rat BMSc were engineered by trans-

gene to express human TH type 2, and GTP cyclohydrolase I (GC), the enzyme providing the  $BH_4$  cofactor for TH.<sup>117</sup> The gene-engineered rat BMSc synthesized and released L-DOPA. When the rat BMSc that synthesized L-DOPA were transplanted into the rat model of PD, L-DOPA was converted to DA metabolites, and behavioral recovery was observed. However, the ameliorative effect of transplanted rat BMSc was short-lived (up to 7 days), presumably because of inactivation of transgenes introduced into the brain with retroviruses. In the experiments, BMSc were transduced sequentially with two separate retroviruses, each containing TH or GC driven by the CMV promoter.<sup>117</sup> In addition, [SVM6] they have created a 3.4 kb bicistronic construct consisting of the TH gene and GC gene separated by an internal ribosome entry site (TH-IRES-GC) to avoid the use of two separate retroviruses.<sup>118</sup> Moreover, a small number of rat BMSc producing L-DOPA continued expressing transgenes after a massive expansion in culture by simple low-density plating of approximately 3 months in vitro. However, the BMSc in these studies did not differentiate into neuron-like cells.

Park et al.<sup>119</sup> demonstrated that retroviral transduction of mouse marrow cells with the GDNF cDNA followed by intravenous 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 weeks before exposure to the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (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 after intrastriatal injection of GDNF.<sup>120</sup>

Dezawa et al.<sup>114</sup> demonstrated the highly efficient and specific induction of cells with neuronal characteristics, without glial differentiation, from both rat and human BMSc using gene transfection with Notch intracellular domain (NICD) and subsequent treatment with bFGF, forskolin, and ciliary neurotrophic factor. MSCs expressed markers relating to neural stem cells after transfection with NICD, and subsequent trophic factor administration induced neuronal

cells. Some of them showed voltage-gated fast sodium and delayed rectifier potassium currents and action potentials compatible with characteristics of functional neurons. Further treatment of the induced neuronal cells with GDNF increased the proportion of TH-positive and DA-producing cells. The cells released a significant amount of DA (1.1 pmol/10<sup>6</sup> cells) after induced depolarization. Transplantation of these GDNF-treated cells showed improvement in apomorphine-induced rotational behavior and adjusting step and paw-reaching tests after intrastriatal implantation in a 6-OHDA rat model of PD.

The risks of virus-associated gene transfer, although low, are not imaginary (given the evidence of secondary malignancy in children after stem cell transplantation). It is also clear that the limited availability of ES cell sources and the uncertainties regarding the safety of therapeutic viral-based gene transfer have generated interest in alternative approaches, including the use of somatic stem cells. We believe that a key ingredient for implementing a regenerative approach is the ability to epigenetically drive stem cell differentiation by *ex vivo* regulation of the local cellular environment rather than by genetic alteration. Modulation of the microenvironment would help provide the framework for multipotent cells to recapitulate tissue growth and organogenesis in a postnatal setting, circumvent the risks of exogenous gene transfer, and may lead to a multifaceted and cost-effective approach with enormous translational implications.

### *Differentiation of BMSc to Dopaminergic-like Neurons In Vivo*

The therapeutic potential of BMSc for the treatment of PD was highlighted by a publication from Li et al.<sup>121</sup> Mouse BMSc prelabeled with BrdU were grafted into the striatum of an MPTP mouse model of PD. The grafted MPTP-treated mice exhibited a significant improvement on the Rotorod test at 35 days after transplant, compared with nongrafted controls. Immunohistochemistry revealed BrdU-reactive cells in the striatum of the grafted MPTP-treated mice at least 4 weeks after transplantation. Double staining showed that approximately 0.8% of BrdU-reactive cells expressed TH

immunoreactivity. Although the mouse BMSc injected intrastrially survive, express TH immunoreactivity, and promote some functional recovery, further investigation is required to understand the mechanism used 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 the marrow-derived cells, mediate the improvement in motor function.

### **Multipotent Adult Progenitor Cells**

Jiang et al.<sup>122</sup> recently described that, similar to mouse ES cells, mouse multipotent adult progenitor cells (MAPCs) can also be induced to differentiate *in vitro* into cells with biochemical, anatomical, and electrophysiological characteristics of midbrain neuronal cells. The population of the MAPCs, a rare cell within human and rat BM mesenchymal stem cultures, can be multiplied more than 120-fold.<sup>123-125</sup> It was demonstrated that cells capable of differentiating *in vitro* to cells of the three germ layers could be selected from rodent BM.

Mouse MAPC differentiation to neurons was achieved after sequential culture for 7 days with bFGF, FGF8, Shh, and BDNF. 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 (AADC and TH), 18% expressed markers of serotonergic (TPH), and 52% of GABAergic (GABA) neurons. Double immunohistochemistry showed that GABA, TPH, and TH were never detected together in the same cell. Mouse MAPC-derived neuron-like cells cultured in the presence of fetal brain astrocytes demonstrated that the cells continued to express markers of dopaminergic neurons (25% TH), serotonergic neurons (25% TPH), and GABAergic neurons (50% GABA) and acquired a much more mature neural morphology with more elaborate array axons.<sup>122</sup> 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 intravenous infusion, and rare donor cells found in the brain did not colabel with neuroectodermal markers.<sup>123</sup>

## Marrow-isolated Adult Multilineage Inducible Cells

D'Ippolito et al.<sup>126</sup> described the marrow-isolated adult multilineage inducible (MIAMI) method. They isolated a population of non-transformed pluripotent human cells from BM with a procedure that was designed to provide conditions resembling the *in vivo* microenvironment that is home for the most primitive stem cells. Marrow-adherent and -nonadherent cells were cocultured on fibronectin, at low oxygen tension (3%), for 14 days and colonies of small adherent cells were isolated and further expanded on fibronectin at low density, low oxygen tension with 2% fetal bovine serum. The cells expressed markers found among ES cells as well as mesodermal-, endodermal- and ectodermal-derived lineages including neural cells. Neural differentiation was induced by exposing the MIAMI cells to NT3, NGF, and BDNF for 3–7 days. At the end of the differentiation, cells expressed NeuN and NF-M, whereas the expression of nestin was not detected, consistent with mature neuronal phenotype. In addition, after a novel neuronal induction protocol, Montero-Menei et al.<sup>127</sup> were able to first obtain cells expressing neural stem cell markers (nestin and  $\beta$ -tubulin III) and at a later stage of differentiation expressing mature neuronal markers such as NeuN and neurofilaments. Moreover, using an improved four-step *in vitro* protocol combining NT3 and retinoic acid together with dopaminergic-inducing molecules (SHH and FGF8), TH-expressing neurons were demonstrated. Moreover, to be able to maintain the differentiated functional phenotype and appropriate number of cells after grafting, a new tool for cell therapy has been developed termed pharmacologically active microcarriers (PAM). PAM assembles biodegradable and biocompatible matrix elements and controlled-release technology that provides the needed environment for supporting the functionality and viability of the implanted cells. The efficacy of the PAM was demonstrated in an animal model of PD using a cell lineage and is currently being studied in this same paradigm with fetal mesencephalic cells conveyed by GDNF-releasing PAM. In the near future, this unique technology will be utilized to produce GDNF-releasing PAM conveying the MIAMI-derived physiologically competent DA-releasing neurons designed for

the sustained clinical improvement of the Parkinson's animal model.<sup>127</sup>

## Possible Mechanisms for Adult BMSc Plasticity into Neurons

What are the proposed mechanisms for adult cell plasticity? Cells have been sorted into unpredicted cellular phenotypes: A. Existence of primitive stem cells in the mature tissue; B. the presence of multiple progenitor/stem cells not derived from the same embryonic germ layers in the tissue; C. direct and indirect dedifferentiation; D. transdifferentiation; and E. cell fusion. It is of essential importance to keep an open mind about these proposals. This does not mean lower scientific standards.

The potential of the BM to differentiate into neurons was first demonstrated in experiments with rodents. Transplanted BM-derived cells were shown to migrate into various brain regions and develop neuron-like features.<sup>128–131</sup> Furthermore, Mezey et al.<sup>132</sup> found Y chromosomes in the human brains of females after transplantation of male BM. 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 may not be general phenomena but may reflect fusion with neurons or transient expression of many proteins including neuronal markers.<sup>133–135</sup> In a recent report, Cogle et al.<sup>136</sup> demonstrated that human hemopoietic cells can transdifferentiate into neurons, astrocytes, and microglia in a long-term setting without fusion. They found that hippocampal cells containing a Y chromosome were present up to 6 years posttransplant in 1% of all neurons and there was no evidence of fusion. In addition, it was demonstrated that rat BMSc were infused into 1.5- to 2-day-old chick embryos.<sup>137</sup> After 4 days, the rat cells had expanded 1.3- to 33-fold in one-third of surviving embryos. The cells engrafted into many tissues, and no multinuclear cells were detected. The most common site of engraftment was the heart, apparently because the cells were infused just above the dorsal aorta. Some of the cells in the heart expressed cardiotin, and  $\alpha$ -heavy-chain myosin. GFP<sup>+</sup> cells reisolated from the embryos had a rat karyotype. Therefore, the cells engrafted and partially differentiated without evidence of cell fusion.<sup>137</sup>

Chopp et al.<sup>144</sup> reported that transplantation of undifferentiated BMSc in rats revealed therapeutic benefit after traumatic brain injury,<sup>138–141</sup> ischemic brain injury,<sup>121,142,143</sup> or spinal cord injury. In these postinjury transplantation studies, generally less than 20% of transplanted cells were immunoreactive for CNS antigens, raising the possibility that the remaining cells contributed to the clinical benefits.

Although many researchers observed the conversion of BM cells into neurons and glia, several fundamental questions remain for future consideration. First, what is the origin of the pluripotent stem cells found in adult BM? Are they related to primordial germ cells that arrive in the tissue early in development and retain the ability to differentiate into neuron-like cells under experimental conditions, or they are rare, but integrate in the population of the adult BM? Second, does the transformation of grafted BM cells into the neuronal phenotype seen in the host tissue occur because of cell fusion between the marrow and host cells or by real transdifferentiation? Replacing patients' BM in the case of blood disorders is well established. However, the clinical application for neurodegenerative diseases such as PD, raises serious questions concerning the safety of the implantation and the competence of the transplanted cell to function as controlled and efficient dopaminergic cells. The use of induced BM-derived neurons for transplantation in animal models of PD is very limited, although several groups reported the improvement in rotational behavior after brain implantation. Thus, although serious basic and clinical studies should be continued, it is not too optimistic to believe that BM-derived stem cells, obviating ethical debate, may provide a therapeutic tool for tissue replacement in PD.

## Future Strategies

In establishing stem cells as an alternative graft source, logistical, 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, offering the possibility of 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, the use of adult stem cells will not be subject to the ethical concerns that surround the use of fetal tissues, including the ES cells.<sup>145</sup> 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 progress is to be made beyond the level of clinical phenomenology. There are three main challenges. First, it will be necessary to learn much more about neuronal development, in order 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 – in order 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; in other words, 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 provided in this review serve to demonstrate the numerous issues in cell biology involved in advancing to a functionally valuable therapeutic strategy in PD. However, the most 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 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. The best stem cell source for generating new DA neurons is not yet known. However, we believe that adult stem cells such as

BMSC are the best cells to use in autologous transplantation in the treatment of PD.

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[SVM1]: Is spelling tetrahydropyridine?

[SVM2]: Please check spelling.

[SVM3]: Should this be plural (patients) as in previous sentence?

[SVM4]: McKay is not listed as one of the authors in the article referenced as 66. Please verify.

[SVM5]: Please check meaning of sentence.

[SVM6]: Please define CMV.

[SVM7]: Please provide page numbers.

[SVM8]: Please provide page numbers