

Developing Novel Cell Sources for Transplantation in Parkinson's Disease

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ABSTRACT

Developing dopaminergic (DAergic) neurons that originate from aborted human embryos have been implanted into the brains of patients with Parkinson's disease (PD) and, in some cases, have successfully restored function. However, there are insufficient numbers of cells available to allow this therapy to become widely used. The limited amount of tissue from embryos may be circumvented by the use of cell lines that can be expanded in vitro for banking, then differentiated into DAergic neurons just prior to implantation into patients. Today, there are four main sources for such cell lines with future potential for banking and cell therapy for PD: human embryonic stem cells, human neural stem cells, human genetically immortalized stem/progenitor cells, and human adult-derived non-neural stem cells, such as bone marrow-derived stem cells. Currently, it is not possible to utilize these cell sources therapeutically for PD. The primary reasons are because it has not been feasible to effectively differentiate these cells into DAergic neurons and because the stability of phenotypic expression has been variable. This chapter describes methods to generate cells suitable for transplantation in PD in the future. The development of novel cell sources is described, along with an overview of the various types of stem cells that are suitable for grafting in PD.

Key Words: Parkinson; transplantation; dopamine; embryonic stem cells; neural progenitor; differentiation; immortalization.

INTRODUCTION

In the central nervous system, the most abundant source of dopaminergic (DAergic) cell bodies is located in the midbrain, mainly in the substantia nigra pars compacta (SNpc), the ventral tegmental area, and the retrorubral

field. These DAergic neurons extensively innervate the forebrain (e.g., projections to the caudate nucleus, putamen nucleus accumbens, olfactory tubercle) and several cortical and limbic regions, including the amygdala, lateral septum, and ventral hippocampus. The basic organization of midbrain DAergic neurons and their projections is consistent across most mammals (1,2).

The DAergic projections originating in the midbrain and innervating the striatum are the most extensively studied catecholamine neurons, partly because the degeneration of DAergic neurons in the SNpc is one cause of the motor dysfunction present in Parkinson's disease (PD). This disorder is characterized by tremor, rigidity, hypokinesia, and postural instability. Levodopa treatment initially provides marked symptomatic relief; however, within 5–10 yr, most patients exhibit a gradual loss of efficacy taking levodopa that is associated with the appearance of involuntary movements (dyskinesias; 3).

Cell Therapy for PD: Proof of Concept

Both neural transplant studies performed in animal PD models and clinical grafting trials have suggested that cell replacement therapies may be effective in treating PD. Proof of this concept was observed in open-label trials more than a decade ago, when transplantation of human embryonic ventral mesencephalic tissue containing DA neurons was shown to be an effective therapy (4–6). The strategy has been to replace the population of degenerated DAergic neurons with neurons harvested from a donor at an early stage in development, when the cells are still dividing and able to grow processes to their appropriate targets in an adult host brain. This strategy is particularly suitable to explore in PD, since its main pathology is relatively focused on the nigrostriatal DAergic system (i.e., a specific neuronal population within a restricted area of the brain). The classical biochemical deficit in PD results from the loss of DAergic neurons in the SNpc. However, there is also degeneration of the acetylcholine and noradrenaline systems innervating the forebrain (7). This neuropathology may contribute to cognitive and other nonmotor features of this disease, some of which are relatively unresponsive to DAergic replacement therapy (8). Grafts specifically designed to replace the loss of DA in the striatum only treats the core pathology of PD. Nevertheless, this may be sufficient to alleviate the most disabling symptoms.

Thus far, over 300 patients worldwide with PD have received transplants of primary human embryonic tissue that is rich in DAergic neurons. Clinical improvements have been reported for up to 10 yr after transplantation surgery (9,10).

Challenges in the Cell Therapy Strategy

Currently, cell-based therapies for PD face three major problems. First, recent placebo-controlled trials have shown levodopa-independent dyskinesias in a minority of patients receiving embryonic mesencephalic tissue transplants (11–13). The cause of the dyskinesias remains unknown, but the placement of transplants into potential hotspots in the dorsal and ventral part of the striatum may lead to the severe side effects (14). In addition, the heterogeneous cellular composition of ventral mesencephalic grafts has also been suggested to underlie dyskinesias. Thus, selective transplantation of DAergic neurons from the SNpc has been thought to increase symptomatic relief and decrease motor side effects, compared to the grafting of DAergic neurons from the SNpc and adjacent ventral tegmental area (15). However, this hypothesis has still not been addressed experimentally in an effective manner.

The second major problem facing scientists in the neural grafting field is that the two published studies on transplantation in PD following a double-blind placebo-controlled protocol did not show a significant improvement when compared with sham surgery (11,13). For unknown reasons, the results differ from the earlier positive reports emanating from open-label trials (9). Naturally, the differences between the two types of studies could be explained by a combination of the placebo effect and observer bias that occurred in the open-label trials. However, there are additional variations in surgical technique, patient selection, and immunosuppressive regimens that may have had equally important roles.

Third, there are significant practical and ethical problems associated with using embryonic/fetal tissues. Thus, one major restriction of the more widespread application of clinical transplantation is the limited availability of suitable human donor tissue, along with a poor survival of DAergic cells in the grafts. The human ventral mesencephalic tissue that is dissected from embryos and used for the grafts is only made up of 5–10% of cells that are destined to become DAergic neurons (16); the remaining cells are other neurons, glia, or precursors. Furthermore, only a small fraction (approx 10%) of those cells that become DAergic neurons actually survive the grafting procedure (17–19).

Due to the problems facing clinical neural grafting in PD, a number of alternative cell sources have been investigated. Cells for transplantation in PD should display the following characteristics:

1. Reliable viability and homogeneity.
2. Availability in large numbers at the planned time for surgery.
3. Ability to differentiate into DA-synthesizing neurons (particularly of the specific phenotype found in the SNpc).

4. Capacity for axonal growth.
5. Genetic stability and lack of tumor formation.
6. Capacity to survive in the host brain for the long term.
7. Long-lasting functional benefit without significant adverse effects.

Another source for implantation in PD is stem cells, defined as undifferentiated cells with high proliferative potential that can generate a wide variety of differentiated progeny (20). They are particularly appealing for cell-based therapies because they can be made available in large numbers, they are relatively easy to maintain in culture and (by definition) exhibit a high differentiation potential, and they could consequently provide the missing DAergic neurons for grafting in PD.

STEM CELLS

Stem cells can be derived from either embryonic or adult tissue sources and are divided into different classes depending on when they are present and on their differentiation capacity. There are two categories based on when they appear during the lifetime of the organism: embryonic stem (ES) cells occur during early embryonic development, and somatic or adult-derived stem cells are present in different tissues in the fully developed organism. It is important to clarify that the term *embryonic stem cells* is usually reserved for cells derived from the inner cell mass of the preimplantation blastocyst (see below). However, other types of stem cells can be derived from later stage embryos that typically have greater lineage restriction. Based on their capacity to differentiate into various cell types, stem cells can be either *pluripotent*, giving rise to every cell of the organism (except the trophoblasts of the placenta), or *multipotent*, giving rise to all the cells of the organ in which the multipotent cell normally resides. As a potential cell source for PD therapy, the most commonly studied somatic stem cells are the neural stem cells derived from embryonic neural tissue.

Somatic Neural Stem Cells

Embryonic Neural Stem and Progenitor Cells

Embryonic neural stem and progenitor cells are obtained from embryonic neural tissue and are capable of self-renewing and generating neurons and glia. Over the past decade, research has shown that it is possible to select, epigenetically manipulate, and genetically engineer cells in culture prior to intracerebral transplantation (21–25). Neural stem cells have been isolated from various parts of the brain, such as the midbrain and forebrain (26,27). These cells can be grown as free-floating cell cultures in so-called *neurospheres*, where they will expand in number. These cells appear to retain

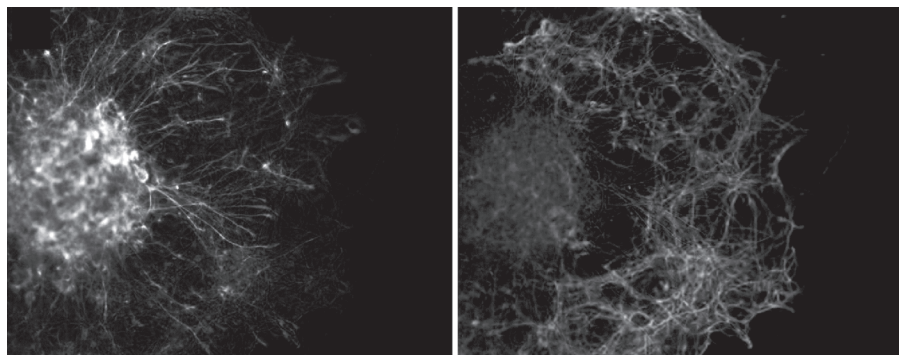


Fig. 1. Three-day differentiation of E14.5 rat ventral mesencephalic neurospheres. Neurons were stained for β -III-tubulin (red) and astrocytes for glial fibrillary acidic protein (GFAP) (green), and they were counterstained using the nuclear marker Hoechst (blue).

their multipotency and the ability to develop along different progenitor, neuronal, and glial lineages under specific culture conditions (28; see Fig. 1).

However, it seems that neuronal differentiation potential differs between animal species and also because of the duration of cell propagation in culture. Human neurosphere cultures, which have been expanded for long periods (up to 150 d), can retain their ability to generate neurons with a high (40%) frequency (23). In contrast, less than 5% of the cells from mouse neurospheres that have been passaged a few times (over 2 wk) appear capable of differentiating into neurons (29). In the case of rat neurosphere cultures, the proportion of cells that differentiate into neurons decreases from around 63% to 17% following three passages over 3 wk (30).

DAergic Differentiation by Epigenetic Factors

As mentioned above, the differentiation potential of an embryonic neural stem cell culture depends on the age of the donor tissue, the precise anatomical region dissected, and the length of time spent in culture (31). This is also the case when differentiating these cells into DAergic neurons. Clearly, it is difficult to influence the differentiation of embryonic neural stem cells and coax them into adopting a DAergic neuronal phenotype. Thus far, the addition of various cytokines and erythropoietin to the culture medium and cell growth in low-oxygen tension have led to the most efficient production of DAergic neurons in vitro (32,33). Expanded embryonic neural stem cells obtained from the ventral mesencephalon have also been transplanted successfully into hemiparkinsonian rats and have demonstrated functional improvement in reducing amphetamine-induced motor asymmetry in recipi-

ents (25,27,34). This procedure could offer a powerful strategy for generating large numbers of DAergic neurons for transplantation, but the techniques to ensure good posttransplant survival are still not well established (25,34). When comparing the numbers of surviving DAergic neurons when mesencephalic tissue is grafted with or without prior expansion in vitro, it appears that there are no major benefits of proliferating and differentiating the precursors in vitro before transplantation (35). This may be explained by the cells' higher susceptibility to transplantation-related death after the period in culture (25,35). Another study suggested that as many as around 75% of rat mesencephalic progenitors can be induced to express TH by treatment with a cocktail of cytokines, but following grafting, the majority of these cells appear to either die or suppress the DAergic phenotype (34). The failure to maintain all the DAergic neurons after transplantation may indicate that when dealing with stem cell-derived neurons, this phenotype is not cell-autonomous but requires external cues or a more permissive environment.

DAergic Differentiation by Genetic Factors

Specific genes involved in the differentiation of DAergic neurons have been artificially expressed in embryonic neural stem cells to direct the differentiation into DAergic neurons. Most successfully, the orphan nuclear transcription factor Nurr1 has been used to engineer neural stem cells to differentiate into DAergic neurons (36). Nurr1 is known to be implicated in the differentiation of mesencephalic DAergic neurons (37). Thus, mice that are null-mutant for Nurr1 lack mesencephalic DAergic neurons (38). Furthermore, ventral mesencephalic and cortical progenitors transduced with Nurr1 by viral vectors have been found to exhibit DAergic characteristics. Nurr1-transduced cells have shown to acquire immunoreactivity for several markers associated with DAergic neurons and were found to synthesize and release dopamine in vitro (36). However, when these cells were transplanted into the striatum of rats with unilateral 6-hydroxydopamine lesions, there was no reduction of apomorphine-induced motor asymmetry. The lack of behavioral recovery was interpreted to be associated with immature neuronal morphologies and low survival of TH-immunopositive cells in vivo (36).

Immortalized Neural Progenitor Cell Lines

Neural stem and progenitor cells are not ideal for cell banking because their mitotic competence is limited (39). They exhibit a proliferative response to mitogens for only a limited time until they approach their natural senescence. Typically, a neural progenitor cell will undergo a number of divisions, then differentiate or undergo cell death (40). As an adjunct to treatment with mitogens, it is possible to obtain large numbers of neural

stem cells and progenitors by immortalizing them through genetic engineering (41). The immortalizing gene arrests the cells at a certain developmental stage and prevents terminal differentiation (42).

Most commonly, immature neural cells are immortalized using a retroviral vector that encodes the propagating-enhancing v-myc protein (43–45). Interestingly, v-myc expression may be involved in telomerase activation, because telomerase is a direct target gene regulated by v-myc (46). Maintaining telomere length is known to have an important role for continued cell proliferation (39,47–51). V-myc propagated neural progenitor cell lines include C17.2, H6, HNSC.100, and MesII, which are derived from developing mouse cerebellum (43,44), 15-wk-old human fetal telencephalon (52), 10-wk-old human embryonic forebrain cultures grown as neurospheres (53), and 8-wk-old human embryonic ventral mesencephalon (54), respectively. By constitutively expressing such oncogenes as v-myc, cell lines proliferate indefinitely in culture; however, they still depend on mitogens (e.g., basic fibroblast growth factor [bFGF], epidermal growth factor, or serum) to divide. In the absence of mitogens, they exit the cell cycle and differentiate (52,53,55). As an alternative to developing cell lines by constitutively expressing oncogenes, some groups have taken advantage of a tetracycline-controlled gene expression system (56,57). This is how the human mesencephalic progenitor cell line MesII was generated (54). MesII cells can differentiate and exhibit neurite extension, generate action potentials, express TH and the DA transporter, and produce DA (see Fig. 2A). This requires specific culture conditions that include the addition of tetracycline, dibutyryl cyclic adenosine monophosphate (cAMP), and glial cell line–derived neurotrophic factor (GDNF) to the growth medium (see Fig. 2B).

Although a tetracycline-regulated v-myc vector design is not safe for clinical application, MesII cells provide an interesting experimental tool and a proof of principle that human DAergic cell lines can be generated and illustrate that immortalized cell lines could be relevant for stem cell therapy for PD.

Another method to generate neural progenitor cell lines for PD is to overexpress Nurr1 in existing neural stem cell lines. Nurr1 overexpression in the C17.2 stem cell line has been shown to promote the differentiation of the stem cells into TH-positive neurons when they are cocultured with type 1 astrocytes from the ventral midbrain (58). The identity of the astroglial factor is unknown, but it is evidently produced specifically by astrocytes in the developing ventral midbrain, because glial cells from other regions do not promote differentiation of the C17.2 cells into DAergic neurons. In a more recent study, cells resembling midbrain DAergic neurons were

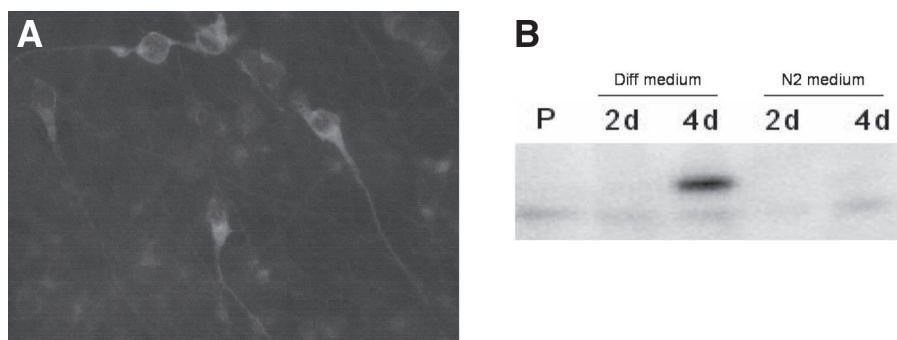


Fig. 2. Immortalized mesencephalic progenitor cells generate TH-positive neurons. **(A)** MesII cells expressing TH immunostaining (red) after differentiation. Cells were counterstained using the nuclear marker Hoechst (blue). **(B)** Western blot of MesII cells grown under specific culture conditions that include the addition of tetracycline, dibutyryl cAMP, and GDNF to the growth medium, which result in TH expression in MesII cells.

obtained from an immortalized multipotent neural stem cell line by overexpressing Nurr1, fibroblast growth factor-8 (FGF-8), and sonic hedgehog (Shh; 59). Shh and FGF-8 are known to direct the differentiation of mesencephalic DA neurons during development (60).

Several immortalized neural cell lines have been examined in transplantation studies. The oncogene must be sufficiently downregulated in the differentiating neural cells, to allow the differentiation programs to proceed and to avoid tumor formation after grafting. Ideally, proliferation should end, and differentiation should be initiated, before the cells are harvested for transplantation. Another option is to rely on the oncogene being spontaneously downregulated in conjunction with graft surgery. V-myc has been reported to be downregulated following intracerebral transplantation (52,61), but detection of *v-myc* mRNA is still possible using reverse transcriptase-polymerase chain reaction (62). This degree of downregulation is unlikely to be sufficient to meet the level of safety required in clinical trials. Nevertheless, in experimental animals, cell lines generated by the constitutive expression of immortalizing oncogenes have been shown as nontumorigenic (63). Following transplantation into neurogenic regions (e.g., the hippocampus), HNSC.100 cells stop dividing after 2 d and spontaneously generate neurons, astrocytes, and oligodendrocytes (62,64). However, results were different when the cells were transplanted into the striatum and substantia nigra of the adult intact rat brain. In these sites, which pertain to neural replacement in PD, cells from the HNSC.100 line displayed less neu-

ronal differentiation in vivo than in vitro and did not generate DAergic neurons (62).

An immortal source of cells can supply unlimited numbers of homogeneous cells for research purposes. They are usually relatively easy to modify genetically and can be readily characterized in detail if they were ever candidates for clinical use. However, there is always the potential risk of creating transformed cells that completely lack growth control mechanisms. Such cells have no contact inhibition, can grow in soft agar, and give rise to tumors in the nude mouse. Their inability to respond to normal signals to withdraw from the cell cycle make them poor candidates for clinical use. To provide an extra level of assurance, it would be prudent to engineer cells with a CRE-loxP recombinase system to remove the immortalizing genes just prior to, or just following, implantation (65). Even if genetically modified cell lines are never used in clinical studies, they can be valuable experimental tools. For example, genetically modified neural stem cells can be compared to neurospheres grown in a mitogen-enriched medium to provide more insight into the key genetic and molecular events that govern neural stem cell differentiation.

Embryonic Stem Cells

First described in 1980 (66), ES cells are pluripotent and form the inner cell mass from the blastocyst stage of all mammalian embryos (67). In addition to ES cells, there are other embryo-derived stem cell lines considered to be pluripotent and immortal: embryonic germ cells and embryonic carcinoma cells. Although embryonic germ and carcinoma cells can develop into a wide range of cell types in vitro and in vivo, ES cells have been shown to differentiate into the widest range of cell types.

To derive ES cells, the isolated inner cell mass is plated on embryonic fibroblasts and is grown in a culture medium supplemented with fetal bovine serum. Alternatively, human ES cells can be grown in a serum-free medium (68,69). ES cells express high levels of telomerase activity, i.e., they are not prone to senescence and are therefore suitable for long-term culture (70). To date, most work has been done on murine ES cells; however, in the last 6 yr, human ES cells have been isolated and cultured (71–73). This chapter describes how ES cells can be differentiated into DAergic neurons and the specific scientific challenges that will be encountered before they can be considered for grafting in patients with PD.

DAergic Differentiation of ES Cells by Soluble Factors

Many studies have been aimed at directing the differentiation of ES cells into DAergic neurons in vitro. Essentially, there are three successful methods for the differentiation of mouse ES cells into DAergic neurons (see Table 1).

Table 1
Protocols Used to Differentiate ES Cells from Mouse and Human Sources into DAergic Neurons

Author and year	ES cell sources	Key features			Graft survival
		of cell differentiation method	TH+ neurons	in vitro	
Bjorklund, L. M., et al. (87)	Mouse ES cells	No special protocol. Cells dissociated prior to grafting	ND		2059 TH+ cells; no survival in 24% of rats ^a
Lee, S. H., et al. (74)	Mouse ES cells	Grown as EBs; exposed to bFGF, Shh, FGF-8, AA	5% ^b		ND
Kim, J. H., et al. (82)	Mouse ES cells overexpressing Nurr1	Transduced with the <i>Nurr1</i> gene; grown as EBs; exposed to bFGF, Shh, FGF-8	56% ^c		4% of total number of implanted cells
Shim, J. W., et al. (84)	Mouse ES cells overexpressing Bcl-XL	<i>Bcl-XL</i> gene; grown as EBs; exposed to bFGF, AA	30.9% ^d		3.6% of total number of implanted cells
Kawasaki, H., et al. (76,77)	Mouse or primate ES cells cocultured with PA6 stromal cells	Grown on PA6 cells	16% and 9%		Mouse: 3% of total number of implanted cells; Primate: ND
Barberi, T., et al. (78)	Mouse ES cells cocultured with MS5 stromal cells	Grown on MS5 cells; exposed to Shh, FGF-8, bFGF, BDNF, AA	?		10–20% of total number of implanted cells
Park, S., et al. (79)	Human ES cells	Grown as EBs; exposed to bFGF, TGF- α	20% ^e		ND
Perrier, A. L., et al. (80)	Human ES cells cocultured with MS5 stromal cells	Grown on MS5 cells; exposed to Shh, FGF-8, BDNF, GDNF, TGF- β 3, dbcAMP, AA	19.2–39.5%		ND

^aNo information about the number of implanted cells. ^bPercentage of TH+ neurons out of total number of cells (6.9% of 71.9%). ^cPercentage of TH+ neurons out of total number of cells (based on ref. 74; 78% of 71.9%). ^dPercentage of TH+ neurons as described in ref. 84. ^ePercentage of TH+ neurons as described in ref. 79. ^fPercentage of TH+ neurons out of total number of cells (64% of 30–79% of 50%). ND, not determined. For explanation of abbreviations, see main text. ?, not determined.

The first method is based on a five-step culture procedure (74) in which the *in vivo* neural development is mimicked by sequential exposure to epigenetic signals. The first step is to generate aggregates called *embryoid bodies* (EBs) from ES cells by dissociating the ES cells into single-cell suspension in a medium containing leukemia-inhibiting factor (LIF) and serum. The EB is an aggregate that can be maintained in a suspension culture and contains precursor cells from all three germ layers (75). The EBs are formed after 4 d, then plated onto an adhesive tissue culture substrate in a medium containing LIF and serum. Neural progenitor cells are then selected by growing the cells in a serum-free medium, and these cells are grown in the presence of bFGF, Shh, and FGF-8. Withdrawal of these factors and the addition of ascorbic acid (AA) induce the final differentiation into DAergic neurons (approx 5% of the total cell number).

The second method designed to obtain DAergic neurons from ES cells involves the differentiation of ES cells on the stromal cell line PA6 (76). The PA6 cell line has been shown to release and/or possess cell surface factors that direct the differentiation of mouse ES cells into DAergic neurons. These factors are still unknown and have collectively been named *stromal cell-derived inducing activity* (SDIA). After being grown on top of PA6 cells, about 16% and 9% of mouse and primate ES cells, respectively, develop into TH-expressing neurons (76,77). These TH-immunopositive cells release DA upon depolarization by high-potassium stimulation.

The third technique also involves stromal cells; in this case, the MS5 cell line (78). Using this cell line, mouse ES cells can be differentiated into neural stem cells. These progenitor cells are then differentiated into DAergic neuron progenitor cells by the addition of Shh and FGF-8. Following the generation of DAergic neuron progenitor cells, bFGF induces further cell proliferation. Subsequently, the withdrawal of these factors and the addition of ascorbic acid and brain-derived neurotrophic factor (BDNF) results in the differentiation into DAergic neurons.

Regarding directed differentiation of human ES cells into DAergic neurons, only two studies have been published thus far (79,80; see Fig. 3 and Table 1).

The first method involves the formation of EBs, followed by the selection of neuronal stem cells by incubation in a serum-free insulin/transferrin/selenium/fibronectin medium and expansion of neural stem cells in the presence of bFGF (79). In the final step, DAergic neurons are enriched by the removal of bFGF and addition of transforming growth factor- α (TGF- α). Following 21 d in the presence of TGF- α , about 15% of the differentiated human ES cells in culture express TH and release DA.

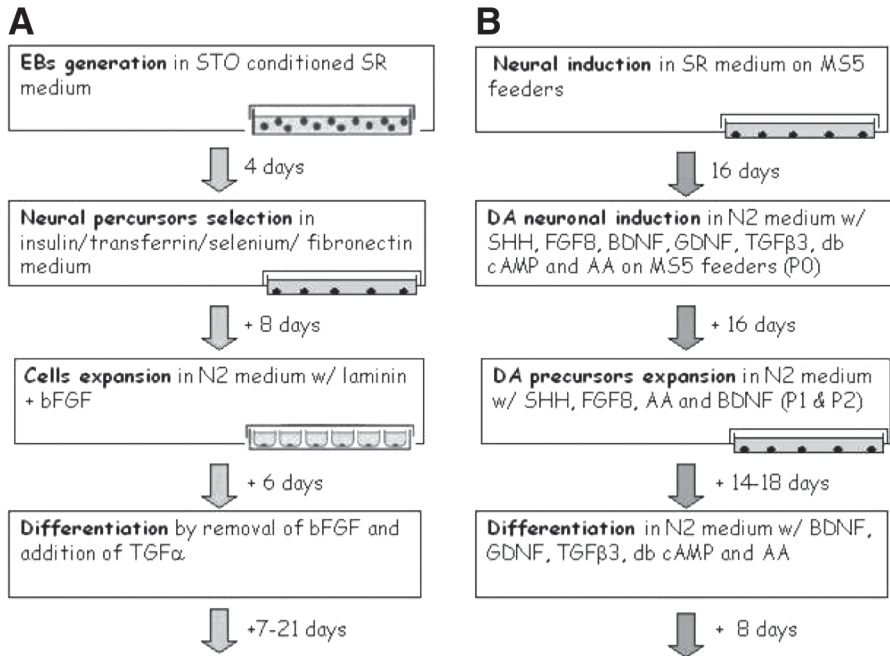


Fig. 3. The in vitro differentiation methods used to generate DAergic neurons from human ES cells. **(A)** ref. 79 and **(B)** ref. 80. SR, serum replacement; P0, P1, P2, passages 0, 1, and 2.

The second method is an adaptation of the previously published method for the DAergic neuronal differentiation of mouse ES cells (78). The first step of the 50-d differentiation procedure is an initial neural induction on stromal cells, followed by neural stem cell expansion and differentiation by sequential exposure to Shh, FGF-8, BDNF, GDNF, dibutyl cAMP, AA, and TGF- β 3. This factor is expressed in early embryonic structures in which midbrain DAergic neurons develop, and it appears to be essential for both the induction and survival of this type of neurons in vitro and in vivo (81). This protocol has been tested on three different human ES cell lines. Interestingly, the cell proportions that differentiate into TH-immunopositive neurons, expressed as a percentage of the β -III-tubulin expressing cells, are both relatively high and similar: ~64%, ~70%, and ~79%.

DAergic Differentiation of ES Cells by Genetic Engineering

As described in an earlier section, knowledge of the genetic control of developing DA neurons is rapidly advancing and is now being applied to ES cell research. Stable Nurr1 overexpression in mouse ES cells has been shown

to increase the proportion of TH-immunopositive neurons from 5% to 50%, expressed as a fraction of the β -III tubulin-immunopositive population. Treatment with Shh and FGF-8 at early stages of the differentiation increases this percentage further to 78% (82). In a similar study, the combined therapy of Nurr1-overexpressing ES cells with AA, Shh, and FGF-8 increased the proportion of TH-positive neurons from 14–61% (83).

To optimize DAergic differentiation and minimize cell death, anti-apoptotic genes (e.g., Bcl-XL) have been overexpressed in ES cells (84). This overexpression of Bcl-XL in mouse ES cells increased the estimate of TH-immunopositive neurons from 18.4% to 30.9% (84).

In another study, a human ES cell line was genetically modified to express TH and guanosine triphosphate cyclohydrolase I—two enzymes necessary for DA synthesis (85). Thus, the endogenous capacity of the ES cells to differentiate into DAergic neurons was not utilized; instead, they were used as vehicles to express the DA-synthesizing machinery. These genetically modified ES cells could produce levodopa *in vitro* (85). When they were transplanted into the striatum of hemiparkinsonian rats with unilateral 6-hydroxydopamine (6-OHDA) lesions of the nigrostriatal pathway, the cells were reported to express TH immunoreactivity and elicit a 46% reduction in the apomorphine-induced rotational behavior up to 6 wk after transplantation, compared to the rotational behavior before transplantation (85). However, there was no evidence of DA production *in vivo*, and functional assays other than drug-induced rotation score were not performed.

DAergic Differentiation of ES Cells In Vivo

The most stringent approach to test whether ES cells have differentiated into true DAergic neurons is to graft them to the adult brain of animals with DA-depleting lesions. In this frequently employed paradigm, it is possible to determine if they can undertake behavioral functions normally associated with DAergic neurons.

Owing to their pluripotency, undifferentiated ES cells transplanted into immunosuppressed mice tend to generate lethal teratomas that contain a wide variety of somatic cells from all three germ layers (86). Surprisingly, a significant proportion of mouse undifferentiated ES cells dissociated into single-cell suspensions has been reported to differentiate into DAergic neurons when grafted to the striatum of immunosuppressed rats (87). Such grafts can even restore parkinsonian symptoms in hemiparkinsonian rats (87). The mechanisms that cause these undifferentiated ES cells of mouse origin to adopt a DAergic neuronal phenotype when transplanted at low density are not understood. Neuronal differentiation has been suggested to represent a “default” pathway of differentiation for mouse ES cells (88). The theory is

that the lack of cell–cell contacts that might stimulate the ES cells to adopt other differentiation protocols would be absent in the single-cell suspensions. Consequently, the cells would only differentiate along the default pathway. However, among 25 rats receiving transplants of 1000–2000 ES cells, lethal teratomas were seen in five rats, and six rats had no surviving grafts at all (87). Clearly, single-cell suspensions do not lead to exclusively DAergic neurons. In addition, although these results show that mouse ES cells can develop into DAergic neurons in the environment of the lesioned striatum, these data also demonstrate that a cell-based therapy for PD cannot rely on undifferentiated stem cells because of their tendency to form teratomas.

When mouse ES cells that had been grown on PA6 cells (i.e., exposed to SDIA) were implanted into the striatum of mice with 6-OHDA lesions, surviving DAergic neurons were observed 2 wk postgrafting. However, only a minority (3%) of the implanted cells were TH-immunopositive neurons (76,89). Following transplantation of mouse ES cells differentiated by being grown on top of the bone marrow stromal cell line MS5 and then exposed to a cocktail of cytokines (Shh, FGF-8, BDNF, and AA) into the striatum of a mouse PD model more than 70% of the TH-positive cells survived. These grafted cells caused a reduction greater than 70% in the drug-induced motor asymmetry in the mice (78).

Hemiparkinsonian rats grafted with cells derived from the *Nurr1*-overexpressing mouse ES cells showed marked behavioral improvement in several tests of drug-induced and spontaneous motor function (82). Interestingly, the most efficient differentiation into DAergic neurons (80% of all cells exhibiting neuronal markers) was achieved when the mouse ES cells were both engineered to overexpress *Nurr1* and were exposed to a combination of Shh and FGF-8 (82). A particularly impressive aspect of this study was that the grafts reversed deficits in spontaneous behaviors (e.g., in the paw-reaching and -stepping tests), as opposed to just drug-induced rotation. In addition, the grafted TH-immunopositive neurons were found to display electrophysiological characteristics consistent with a DAergic phenotype. In another study, mouse ES cells overexpressing *Bcl-XL* exhibited more extensive fiber outgrowth and supported a more pronounced amelioration of behavioral symptoms than transplanted wild-type ES cells (84). These observations were similar with earlier findings showing that overexpression of *Bcl-2*, another related antiapoptotic molecule, increases the extent of their fiber outgrowth into the host brain in grafted primary mouse midbrain DAergic neurons (90,91).

As mentioned in the DAergic Differentiation of ES Cells by Soluble Factors section, it is possible to derive DAergic neurons from human ES cells in

vitro. However, successful integration and survival of transplanted DAergic differentiated human ES cells have not been reported yet. Thus, an important future step is to examine under what conditions human ES-derived DAergic neurons are able to survive, integrate, and function when transplanted into the rat and the primate models of PD.

ALTERNATIVE NON-NEURAL STEM CELL SOURCES

Somatic stem cells have been claimed to possess a broad differentiation potential, and several studies over the past 5 yr have suggested that stem cells from tissues outside of the brain are also capable of generating neurons (92). Such stem cells may be considered alternatives in PD, and this chapter describes two potential sources: bone marrow-derived cells (93–95) and umbilical cord stem cells (96). Other potential sources include adult multipotent progenitor cells derived from bone marrow (97) and skin-derived stem cells (98), but only a few studies on these cell sources have been published to date.

Bone Marrow Cells

Adult bone marrow cells have been recently proposed as an alternative source of neural donor tissue. Unlike ES cells, which are derived from the inner cell mass of a blastocyst, or neural stem cells isolated from different brain regions, adult bone marrow cells are divided into two subpopulations: hematopoietic and bone marrow stromal cells. The bone marrow-derived stem cells are interesting to mention in the context of cell therapy for PD, as it has been repeatedly suggested that they can generate DAergic neurons. When differentiated, hematopoietic stem cells normally give rise to myeloid and lymphoid cells (99), whereas bone marrow stromal cells differentiate into fat (100), tendon and cartilage (101), bone (102) and muscle (103), as well as constituting a microenvironment that is required for the proliferation of hematopoietic stem cells (104). It has not yet been reported whether hematopoietic stem cells (short- and/or long-term renewal progenitors), characterized by specific cell surface markers (i.e., lineage-negative, c-Kit-positive, and Sca-1-positive), can differentiate into lineages other than blood cells. However, unsorted bone marrow cells may be capable of differentiating into neural cells in vitro (93,105,106).

Furthermore, two landmark studies in 2000 suggested that bone marrow cells could differentiate into neurons in vivo (107,108). In these studies, infusion of bone marrow stem cells into neonatal or irradiated adult mice showed that small fractions of neuronal cells (0.3–2.3%) in the central nervous system contained donor cell markers. However, these findings have

been questioned by a series of follow-up studies, because bone marrow stem cells have been shown to adopt the functional features of neural lineages via cell fusion, rather than by signal-mediated differentiation (109–112). Another study based on bone marrow transplantation has highlighted the possible transdifferentiation potential of bone marrow stromal cells (113). Yet, the current consensus is that there is insufficient evidence that bone marrow stem cells can express neuronal markers upon transplantation, only that they can differentiate into microglial cells after transplantation (109,111,114).

A recent study suggested that engineered human bone marrow cells could be suitable for autologous transplantation in PD (94). In this study, human bone marrow cells transfected with the Notch intracellular domain protein and treated with neurotrophic factors differentiated into TH-expressing neurons. Transplantation of these TH-expressing neurons into the striatum of 6-OHDA-lesioned rats resulted in reductions in apomorphine-induced motor asymmetry (94), but effects on spontaneous behavior were not shown, and there was no direct evidence of production and release of DA. Because apomorphine-induced rotation can also decrease in response to nonspecific damage of the striatum, these findings did not unequivocally prove that the grafted cells were DAergic. Nevertheless, this initial study definitely warrants follow-up experiments; if bone marrow cells can generate DAergic neurons, it would have tremendous implications for cell therapy in PD. In a similar manner, rat and human bone marrow cells have been transplanted into the cerebral ventricles of 15-d-old rat embryos (115). These cells migrated throughout the developing brain, and after 1 mo they were found to express the neuronal marker NeuN in the cortex and olfactory bulb. From that study, and contrasting previous reports where bone marrow was grafted into the adult brain, the embryonic brain may contain factors vital to the promotion of neuronal differentiation.

Interestingly, considerable overlap of gene expression profiles between ES, neural stem cells, and hematopoietic stem cells has been found (116). This overlap suggests that common pathways exist between different stem cell classes undergoing differentiation. Thus, it is conceivable that a differentiation protocol, established as effective in generating neurons from embryonic and neural stem cells, may be applied to the other classes of stem cells and may stimulate the formation of the same specific neuronal phenotypes.

An alternative explanation for the presence of neural markers in bone marrow-derived cells is that the bone marrow niche contains several different types of committed tissue-specific stem cells. These cells are merely

waiting for a signal indicating that they need to migrate back to the organ where they were derived and differentiate into cells specific for that particular organ (117). Thus, the bone marrow cells prone to neural differentiation may have originated from neural tissue, rather than directly from the bone marrow lineage, and are kept as a reservoir in the bone marrow niche. This could explain why, in response to the mitogens bFGF and epidermal growth factor, some bone marrow cells form neurospheres that give rise to neural cells when differentiated (118) or express neuronal markers prior to differentiation (119). There has been little characterization of these subgroups thus far.

Among all the bone marrow studies, the concept that adult bone marrow stromal cells could be used in the future for brain repair seems to be the most significant. However, the potential appears to lie more specifically in certain cell subgroups located within the bone marrow. A greater focus on these subpopulations may yield more encouraging results than those presently obtained.

Umbilical Cord Blood Cells

Blood cells taken from the umbilical cord have been shown to have the capability to differentiate into both neuronal and glial cell types (96,120,121). Blood in the human umbilical cord contains hematopoietic and mesenchymal stem cells; yet, in contrast to the adult bone marrow, the advantage of using cord blood cells is that the tissue can be obtained at birth and cryopreserved for several years. Such cells could be used as routine starting material for the isolation and expansion of cells for autologous transplantation (120). Thus, blood cells of the umbilical cord could be transdifferentiated into other cell types, human cord blood banks could be created for use in autologous transplantation.

THE OPTIMAL CELL FOR PD THERAPY:

PROS AND CONS WITH SOURCES CURRENTLY AVAILABLE

Embryonic Neural Stem Cells as Cell Therapy for PD

Although DAergic neurons can be generated in large numbers from neural precursors under special conditions (25), they are still not a realistic option as donor cells for transplantation in PD. Limitations in long-term propagation of the cells and difficulties in achieving stable DAergic differentiation continue to be major problems. As mentioned in the DAergic Differentiation by Epigenetic Factors section, the efficiency of DAergic differentiation decreases with time in midbrain neural precursors after proliferation for extended periods in vitro (30). Furthermore, although expanded precursors

can differentiate into DA-producing neurons in vitro, they do not always maintain a DAergic phenotype after intracerebral implantation (31,36,59). Improved understanding of the biology of neural stem cells could reveal more clues about the signals that favor the differentiation of stem cells into dopaminergic neurons and help resolve the puzzling fact that cells that have differentiated in vitro do not always preserve their phenotype.

ES Cells as Cell Source for PD

The time necessary to expand human ES cells into large numbers of human DAergic neurons is relatively long (at least 39 d [79,80; see Fig. 3]). The length of the existing differentiation protocol may be a limitation for a routine production of DAergic neurons (i.e., increased risk of contamination).

The risk that undifferentiated cells are included among transplanted human ES-derived DAergic neurons is a major issue. Obviously, this can lead to the formation of lethal teratomas in the host brain. Chromosomal aberrations identified in mid-term cultured human ES cells are a cause for concern (122). This suggests that long-term propagation of human ES cells can lead to abnormalities in the cells intended to be used for grafting purposes. Eventually, existing human ES cell lines could accumulate chromosomal aberrations and mutations that either inhibit differentiation into DAergic neurons or cause differentiation along unpredictable pathways. Both scenarios would render them highly unsuitable for grafting and require that new “mutation-free” human ES cell lines are derived. The most important current shortcoming with human ES cell lines as a potential source of cells for grafting in patients with PD is the absence of convincing transplantation data in experimental animals. Indeed, the survival after grafting, integration in the host brain, and the release of DA, leading to the decline in motor dysfunctions, remains to be shown by the human ES-derived DAergic neurons in animal models of PD.

Goals to Achieve for Cell Therapy in PD

Importantly, grafts of stem cell-derived neurons should be performed in such a manner that dyskinesias are not triggered in patients with PD. Currently, we do not understand why graft-induced dyskinesias can develop in some patients. Nevertheless, it will probably be possible to graft cells to PD patients that reproducibly never cause dyskinesias. It may require a well-characterized and homogenous source of cells. Most likely, it will be necessary to target these cells to specific host brain regions, and possibly only certain types of PD patients (regarding age of onset, duration of disease, and history of medication), will be suitable to avoid the dyskinesias. Even if the

problem is resolved, stem cell therapy has its own set of issues that need attention. The optimal differentiation stage of the stem cells at the time of transplantation is likely to be an important factor to understand. Final differentiation occurs once progenitors have exited the cell cycle, and it is often characterized by expression of the enzymes or neurotransmitters required for neural function (123,124). We need to determine whether the differentiated ES cells or neural stem cell culture should be harvested *before* or *after* the cells undergo their genetically determined final cell division. Based on the authors' experience grafting primary DAergic neurons obtained from aborted embryos, the optimal time for harvesting might be when they commence a period of vigorous neurite growth directed toward appropriate targets in the developing brain (125). Although there are reasons to believe that predifferentiated cells may be particularly sensitive to the trauma involved in transplantation, evidence also shows that an undifferentiated DAergic progenitor will not develop into a DAergic neuron in the adult striatum (125).

Graft immune rejection is another limitation of cell-based therapies that could be overcome using stem cells. Obviously, if somatic stem cells could be used, it may be possible to isolate the patient's own cells and perform an autograft, which raises no immune issue. Also, human ES-based therapy offers solutions to the immune problems. Creation of human blastocysts via the transfer of the nucleus of a patient's somatic cell to an enucleated oocyte would allow the derivation of human ES cell lines immunocompatible with patient. This is the basis of therapeutic cloning. The procedure is ethically controversial; aspects of the methodology are similar to those used for reproductive cloning—a practice, if applied to humans, considered unethical in most societies. Despite all the ethical discussions around human cloning, a group in South Korea has already published the derivation of a human ES cell line from a cloned human blastocyst (126). This cell line is claimed to be able to give rise to any tissue that would not be rejected by the donor of the somatic nucleus.

Purification of DAergic Progenitors for Cell Therapy in PD

Thus far, ES cells have not been shown to produce a specific, pure population of cells after differentiation. Moreover, some of the ES cells in cultures derived from EBs do not undergo differentiation. Such cells should be removed or eliminated prior to transplantation; otherwise, they could be the source of unwanted cell proliferation. When developing cell therapy for PD, it would be valuable to purify the DAergic cell population using a methodology like fluorescence-activated cell sorting (FACS). Then, it would be possible to graft predetermined, homogenous populations of cells and define

the optimal cell type for grafting. In some studies, another approach has been used in attempts to obtain more homogenous grafts. For example, mouse ES cells have been treated with antimetabolic drugs prior to transplantation to avoid the overgrowth of mesenchymal tissue and tumor formation. Because the DAergic neurons derived from the ES cell cultures are postmitotic, they are not affected by the antimetabolic agent (77).

Currently, there is a lack of an adequate panel of cell surface markers that can help identify cells at different stages of differentiation. Green fluorescent protein (GFP) expression, under the control of specific promoters related to DAergic differentiation, is one method to identify DAergic neurons. Already, labeling of TH-expressing DAergic neurons has been shown to be a simple and useful system for the purification of DAergic neurons by FACS. Using this approach, GFP-fluorescent DAergic neurons can be used for the functional identification of molecules governing mesencephalic DAergic differentiation and for preclinical research, including pharmaceutical drug screening and transplantation (127). Another study has shown successful FACS of mesencephalic DAergic neurons using the Pitx3-regulated GFP reporter system (128). The *pitx3* gene is expressed in mesencephalic DAergic neurons and is involved in the generation of the mesencephalic DAergic phenotype (129). In this case, ES cells were generated from transgenic mice that express GFP inserted into the transcription factor Pitx3. This demonstrates that the natural expression of specific transcription factors can be used as a selection marker for development along the DAergic lineage. Thus, the use of reporter expression systems can be a powerful tool for the purification of differentiating DAergic neurons for transplantation. In the adult central nervous system, expression of the transmembrane protein Δ -like 1 has been observed in monoaminergic nuclei in the adult brain, including SNpc and the ventral tegmental area (130). This suggests that the Δ -like 1 protein could be used to select dopaminergic neurons. If a panel of cell surface markers is developed, human ES-derived DAergic neurons could be sorted based on markers characteristic of nigral DAergic neurons before transplantation. Thereby, it would be possible to enrich for DAergic neurons and to remove unwanted cell material from the cultured cells.

CONCLUSION

The key to restorative stem cell therapy is to induce the differentiation of stem and progenitor cells into the desired phenotype. The elucidation of regulatory cascades influencing the specification and development of neuronal types is essential in embryonic (neural) stem cell research. Although our current understanding of factors influencing DAergic neuron develop-

ment is in its infancy, the field is advancing rapidly. Possibly, the future will show that somatic stem cells can be differentiated into DAergic neurons. As evident from data discussed in this chapter, a great deal is known already about mouse and human ES and neural stem cells and their capacity to differentiate into DAergic neurons. These stem cells may someday facilitate the development of neural transplantation as therapy for PD.

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