Expert Opinion

Ashley Publications
www.ashley-pub.com

Cell- & Tissue-based Therapy

Embryonic stem cells as a cell source for treating Parkinson's disease

Hitoshi Fukuda & Jun Takahashi†
†*Kyoto University Graduate School of Medicine, Department of Neurosurgery, 54 Kawahara-cho, Shogoin, Sakyo, Kyoto 606-8507, Japan*

Parkinson's disease (PD) is a neurodegenerative disease characterised by a loss of midbrain dopaminergic (DA) neurons. Transplantation of DA neurons represents a promising treatment for PD, and embryonic stem (ES) cells are a good candidate source for DA neurons. However, although recent reports have demonstrated that DA neurons can be efficiently induced from ES cells and function therapeutically in an animal model of PD, many problems remain to be solved in order for ES cells to be used for clinical applications. This review will describe the current status of this field and the obstacles yet to be overcome, and will outline future research approaches from the clinical perspective.

Keywords: clinical application, dopaminergic neuron, embryonic stem cell, Parkinson's disease, transplantation

Expert Opin. Biol. Ther. (2005) 5(10):1273-1280

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder characterised by a loss of midbrain dopaminergic (DA) neurons and a subsequent reduction in striatal dopamine [1]. Although initial pharmacological treatment with L-dihydroxyphenylalanine (L-DOPA) has proven effective, the efficacy of this treatment progressively declines and motor complications, such as dyskinesia, may also develop. As a result, alternative approaches such as deep brain stimulation and fetal DA neuron transplantation [2-4] are necessary for the treatment of PD. Studies using animal models and clinical investigations have shown that transplantation of fetal DA neurons can produce symptomatic relief [5-9]. However, the technical and ethical difficulties in obtaining sufficient and appropriate donor fetal brain tissue have limited the application of this therapy.

Embryonic stem (ES) cells are derived from the inner cell mass of the preimplantation blastocyst and possess many of the characteristics required of a donor cell source for transplantation therapy. In particular, they have the remarkable ability to self-renew *in vitro* virtually indefinitely without losing their capacity to differentiate into any cell type of any organ [10]. Although mouse ES cells were first established in 1981 and were used to produce the first gene-targeted mouse in 1988 [10], it was not until 1998 that human ES cell lines were established, drawing wide attention to the potential usefulness of these cells for cell replacement therapy to treat neurological diseases [11]. Indeed, recent studies have shown that DA neurons can be efficiently induced from ES cells [12,13] and can be used effectively in stem cell treatment in animal models of PD [14-16], suggesting that transplantation therapy for PD using human ES cells might be feasible. However, many problems remain to be solved in order for the clinical application of human ES cell therapy to be practical. In general, preparation of ES cell-derived DA neurons for transplantation is carried out in three stages (see **Figure 1**). First, undifferentiated ES cells must be expanded to

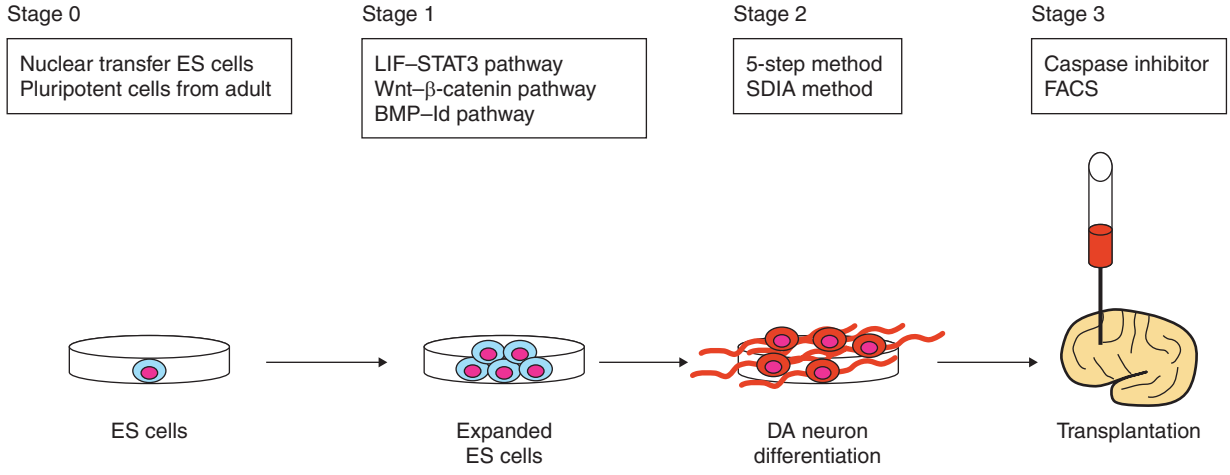

Figure 1. The three critical stages involved in ES cell transplantation therapy for Parkinson's disease. Stage 0: Prior to the three critical stages, determination of donor cells should be considered separately. Nuclear transfer ES cells and pluripotent cells from adult tissues will help accomplish virtually autologous transplantation (see heading 4). Pluripotent adult cells will also contribute to avoiding ethical issues as they can be obtained without destroying human blastocysts (see heading 5). Stage 1: ES cells should be expanded to obtain large numbers of DA neurons, enough to compensate for patients' symptoms. Modulation of the signal pathways shown here may be an alternative for the use of feeder cells and serum, which have been essential for the expansion of undifferentiated ES cells so far, but should be eliminated as chemically undefined factors. Stage 2: Expanded ES cells should be properly induced into functional DA neurons. Methods used so far are generally categorised into two main streams. The step by step (5-step) method is based on the selective expansion of neural precursor cells and induction into specific lineage by exogenous factors. The SDIA method is based on the unknown inducing activity derived from feeder cells. Detailed analysis of molecular mechanisms involved in these methods will contribute to the establishment of human ES cell differentiation systems in chemically defined conditions (see heading 3). Stage 3: In the ES cell-derived graft, undesired cells such as undifferentiated ES cells are contaminated. Selection of desired cell populations using FACS will be helpful to avoid tumour formation as a lethal side effect. To protect the graft from mechanical and chemical damage during transplantation, caspase inhibitors may also be helpful (see heading 4).

BMP: Bone morphogenetic protein; DA: Dopaminergic; ES: Embryonic stem; Id: Inhibitor of differentiation; LIF: Leukaemia inhibitory factor; SDIA: Stromal cell-derived inducing activity; STAT: Signal transducer and activator of transcription.

generate enough differentiated cells. Second, differentiation into DA neurons must be strictly controlled. Third, the transplantation procedure must be optimised in order to maximise survival of the graft without inducing side effects such as tumour formation and dyskinesia. This review will outline and discuss some of the critical issues involved in these three stages, as well as a novel approach for controlling host environments to optimise graft survival and cell integration.

2. Expansion of embryonic stem cells in the undifferentiated state

In 2001, the results of a double-blinded, sham surgery-controlled trial of the transplantation of human embryonic DA neurons into patients with PD were reported [5]. Among patients 60 years old or younger, standardised evaluations of PD revealed a significant improvement in the transplantation group. However, cells from at least four aborted human embryos were required per patient in order to produce symptomatic recovery, making this procedure unfavourable for treating large numbers of patients. In an attempt to increase the supply of DA neurons, rat fetal ventral mesencephalic cells, which include DA neuronal precursors, were expanded *in vitro* and then transplanted into rats with PD symptoms. Although symptomatic recovery was observed, the cell survival rate was as low as 3 – 5% *in vivo*, offsetting any advantage gained by grafting *in vitro*-expanded cells rather than fresh (unexpanded) fetal ventral mesencephalon [17]. These results provided a strong impetus to seek alternative cell sources that could be expanded *in vitro*.

The establishment and maintenance of both mouse and human ES cells has largely been carried out using mouse embryonic feeder cells and serum [18,19]. However, for clinical applications, human ES cells must be propagated and differentiated under chemically defined conditions without any contact with non-human proteins. Mouse ES cells can be effectively maintained without feeder cells in a serum-containing medium when leukaemia inhibitory factor (LIF), which activates the Janus kinase/signal transducer and activator of transcription-3 pathway, is added [20]. In contrast, human ES cells do not exhibit such a response to exogenous LIF and instead require mouse embryonic feeder cells, or at least the conditioned medium of the feeder cells, to remain undifferentiated. Recently, Sato *et al.* examined the role of various signalling

pathways in the self-renewal of human ES cells by performing global expression screens using GeneChips or microarrays. They found that key signalling molecules of the Wnt pathway, which are known to participate in the control of gene expression, proliferation and differentiation, were expressed in undifferentiated human ES cells [21]. The canonical Wnt/β-catenin pathway was then activated by applying 6-bromoindirubin-3'-oxime (BIO), a new pharmacological glycogen synthase kinase-3 inhibitor derived from mollusc Tyrian purple, to both human and mouse ES cells. BIO-treated cells were maintained in an undifferentiated and pluripotent state, as assayed by both cell morphology and expression of genes known to be involved in ES cell maintenance, including the POU family transcription factor Oct-4 and the recently identified homeodomain protein Nanog. Notably, BIO treatment enabled human ES cells to remain undifferentiated in the absence of feeder cells or conditioned medium.

In addition to LIF and Wnt signalling, the bone morphogenetic protein (BMP) family of cytokines has recently been shown to cooperate with LIF to maintain pluripotency of mouse ES cells [22]. Of particular note is the fact that the addition of BMPs is able to replace the effect of serum for these cells, suggesting that it may be possible to culture human ES cells in a serum-free medium. BMPs activate Smad transcription factors, which then turn on the expression of inhibitor of differentiation (Id) genes in neural stem cells [23] and mouse ES cells [24]. Id proteins are negative regulators for basic helix-loop-helix transcription factors that specify various cell lineages [22,23], and overexpression of Id1 in mouse ES cells recapitulates the effect of BMP signalling in the presence of LIF.

In summary, human ES cells can already be grown in an undifferentiated and pluripotent state in chemically defined media, but at this point still require serum for maintenance. However, work with mouse ES cells suggests that if soluble molecules regulating the BMP–Smad–Id pathway or other pathways involved in the maintenance of the stem cell state of human ES cells could be identified, a culture system could be developed that would help make these cells safe for clinical applications.

3. Dopaminergic differentiation from embryonic stem cells

Over the last decade, much progress has been made in unravelling the intricacies of lineage specification of ES cells [25]. ES cells cultured in the presence of serum can be differentiated into aggregates called embryoid bodies, which contain cells derived from all three germ layers [18,26]. However, neural cells represent only a small fraction of cell types formed. When a supraphysiological dose (up to 1 μM) of retinoic acid (RA) is added to the culture medium, relatively high proportions of neural cells are generated from embryoid bodies [27]. However, neurons with midbrain DA character are not generated, possibly because RA treatment causes caudalisation of ES cell-derived neural precursors [28,29].

In 2000, two groups reported methods for generating DA neurons from mouse ES cells, thus opening the way to unlimited *in vitro* production of these neurons. Lee *et al.* [12] introduced a '5-step method' in which undifferentiated ES cells (Step 1) were differentiated into embryoid bodies on non-adherent culture plates for 4 days in the presence of serum (Step 2). The embryoid bodies were then plated on adhesive tissue culture plates for 24 h in the presence of serum and transferred to serum-free medium for 4 – 6 days to select for neural precursor cells (Step 3). These cells were dissociated and grown for 6 days on an adhesive substrate in the presence of fibroblast growth factor (FGF)8, sonic hedgehog and basic FGF (bFGF) (Step 4). Finally, bFGF was removed, and ascorbic acid was added for an additional 6 – 15 days (Step 5). The resulting neurons expressed DA neuronal markers Nurr1 and tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis. Furthermore, these neurons released dopamine in response to depolarisation with potassium. Under optimal culture conditions, 71.9% of the ES cells assumed a neuronal morphology, 33.9% of which were dopaminergic. As cells were grown in chemically defined media, with serum added only at Step 1 and Step 2, this technique may be applicable to clinical therapy in the near future. However, despite extensive work so far, midbrain DA neurons have not been effectively induced from primate or human ES cells using the '5-step method' [30].

Independently, Kawasaki *et al.* reported another method for generating DA neurons from ES cells [13]. In contrast to the '5-step method', this method does not require growth in serum, the formation of embryoid bodies, or the selection of neural precursor cells. Instead, the authors demonstrated that the bone marrow-derived stromal cell line PA6 is a potent inducer of neural differentiation from ES cells. They named this activity stromal cell-derived inducing activity (SDIA). After coculture with PA6 cells for 14 days, 52% of mouse ES cells differentiated into neurons, 30% of which were dopaminergic. Intriguingly, the SDIA method is also applicable to primate ES cells, although the efficacy of neural and dopaminergic differentiation is about half of that observed with mouse ES cells [31]. Recently, Perrier *et al.* succeeded in inducing DA neuron differentiation from human ES cells by modifying the SDIA method [32], which were also confirmed by other investigators [33,34]. Thus, the SDIA method represents a promising step towards the clinical application of human ES cell therapy. However, as PA6 feeder cells are mouse-derived, the molecular mechanisms of SDIA must be elucidated so that the signalling molecules responsible for inducing DA neuron differentiation can be directly applied to ES cells.

In addition, studies have shown that the initial induction of early neural precursors from ES cells is a critical step in neuronal differentiation. As soluble factors regulating the positional identity of neural tissues *in vivo* have already been shown to impart regional character to neuronal ES cell derivatives [15,28,29], future work focusing on the molecular factors

governing early neural induction will be useful, particularly in culture systems in which ES cells adopt neural fates in the absence of serum or feeder cells [35-37]. Indeed, it was recently reported that DA neurons were induced in serum- or feeder-free conditions by modulating early neural induction steps [38,39].

4. Transplantation of dopaminergic neurons derived from embryonic stem cells

Once DA neurons are efficiently generated from ES cells, these cells must be shown to function in the brain to relieve the symptoms of PD. Björklund *et al.* reported that low concentration, single-cell suspensions of embryoid bodies derived from mouse ES cells proliferate and spontaneously differentiate into DA neurons when transplanted into the 6-hydroxydopamine (OHDA)-lesioned rat striatum, and can rescue motor asymmetry defects in PD models [14]. However, they also observed overproliferation of the grafted cells, resulting in teratoma-like tumour formation in 5 (20%) of the 25 recipient animals. As mesodermal, endodermal and even undifferentiated ES cells are contained in embryoid bodies and can cause tumour formation, methods to exclude these non-neural cells must be established in order to avoid tumourigenesis as a side effect of cell transplantation.

In addition, Kim *et al.* observed a significant behavioural improvement in mouse PD models following transplantation of DA neurons generated by the '5-step method' from ES cells overexpressing Nurr1, a transcription factor that plays an important role in the differentiation of midbrain precursors into DA neurons. They observed that ~ 80% of the ES cell-derived neurons were TH-positive, twice the percentage observed using the conventional '5-step method', resulting in 56% of total cells differentiating into DA neurons. When transplanted into the striatum of 6-OHDA-lesioned rats, these DA neurons showed electrophysiological activity *in vivo* and were able to rescue the rotational asymmetry defects associated with ipsilateral degeneration of DA neurons [15]. A similar improvement was reported from the transplantation of ES cell derivatives differentiated by a modified SDIA protocol [16].

As mentioned above, the SDIA method can efficiently induce DA neurons from primate ES cells. Applying these cells to a monkey model of PD, Takagi *et al.* demonstrated that DA neurons induced from monkey ES cells reduce Parkinsonian symptoms when transplanted into animals (see **Figure 2**). After coculture of monkey ES cells with PA6 cell lines for 14 days, the authors detached the ES cell derivatives and cultured them as floating aggregates in the presence of FGF-2 and FGF-20 for an additional 7 days [40]. FGF-20 is a member of the FGF family, which is expressed exclusively in the substantia nigra of the midbrain and is reported to have a supporting effect on midbrain DA neurons [41]. This treatment caused an approximately fourfold increase of TH-positive neurons compared with the conventional SDIA method. A primate model of PD has already been established by repetitive injection of the DA-specific neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which results in many symptoms characteristic of human PD, such as tremor, rigidity, akinesia and gait disturbance [42]. When transplanted into the striatum of MPTP-treated monkeys, monkey ES cells treated by SDIA and FGF-2/FGF-20 survived and functioned as DA neurons, resulting in behavioural recovery and a decrease in Parkinsonian symptoms. Increased uptake of ^{18}F-fluorodopa, observed by positron emission tomography, confirmed that the grafted cells functioned as dopaminergic neurons.

Although DA neurons induced from both mouse and primate ES cells are able to treat Parkinsonian symptoms in animal models, there yet remain problems to be solved. First, ES cells differentiate heterogeneously and do not behave synchronously, resulting in contamination of ES cell-derived grafts with undifferentiated ES cells [13,36]. Transplantation of undifferentiated ES cells or embryoid bodies into the adult brain has been shown to result in the formation of teratomas or teratocarcinomas [14,43]. In most reports so far, teratoma formation was not reported after transplantation of differentiated neural cell grafts derived from mouse and human ES cells [15,44-46]. These studies, however, were xenograft transplantations (for example, mouse cells into a rat host), suggesting that the lack of tumour formation in these experiments might be caused by severe immunorejection due to transplantation of cells from different species. Indeed, allograft experiments in which mouse ES cell-derived neurons were transplanted into a mouse host resulted in tumour formation or tumour-like cell growth even though the cells had been differentiated for $\geq$ 14 days *in vitro*, possibly due to a small fraction of undifferentiated ES cells in the graft [16,43,47]. Therefore, it is essential for undifferentiated ES cells to be eliminated from the graft in order for transplantation to be feasible. Recently, Ying *et al.* reported that ES cells can be differentiated into neural precursors by an adherent monolayer culture, without the formation of embryoid bodies or growth on feeder cells [36]. In this report, the authors used fluorescence-activated cell sorting to purify neural precursors differentiated from a Sox1-GFP knock-in reporter ES cell line. Sox1 is the earliest known specific marker of neuroectoderm in the mouse embryo [48]. Another group has succeeded in purifying ES cell-derived neural precursors by inducing selective apoptosis of undifferentiated ES cells using novel ceramide analogues [49]. Thus, both optical and pharmacological technologies to eliminate undifferentiated ES cells are being developed to support the possible application of human ES cells to transplantation therapy, although further investigation will be required to completely prevent tumour formation as a side effect.

Another problem to be addressed is the low survival rate of the grafted cells in the brain. In animal experiments, the survival rate of transplanted DA neurons is extremely low (0.8 – 5%) [17,40]. This is probably due to the mechanical damage during cell preparation and injection, lack of tropic factors, and even acute inflammation. As apoptosis appears to contribute greatly to cell death following transplantation,

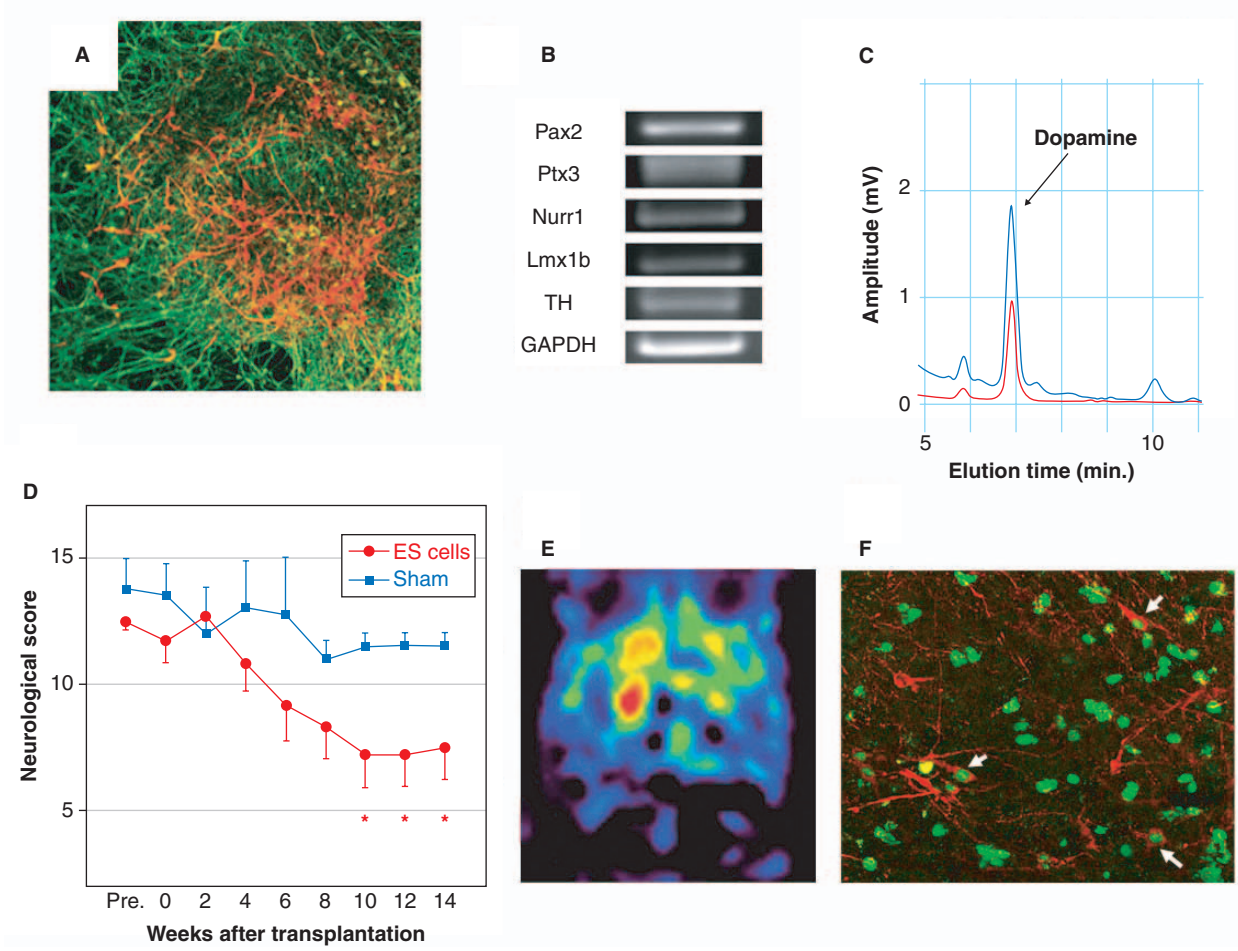

Figure 2. Results from the transplantation of cynomolgus monkey ES cell-derived grafts into MPTP-treated monkey models of Parkinson's disease. (Permission by copyright from the American Society for Clinical Investigation). **A**) Numerous TH-positive DA neurons were generated from cynomolgus monkey ES cells by a SDIA protocol with minor modifications. Red: TH (DA neurons), Green: Tuj1 (postmitotic neurons). **B**) Expression of Pax2, Ptx3, Nurr1 and Lmx1b suggests that grafted cells have a midbrain DA neuronal character. **C**) Dopamine release from grafted cells in response to high K^+ stimuli, assayed by HPLC. **D**) Behavioural scores showed a significant improvement in the transplantation group (n = 6) compared with the sham-operated group (n = 4). **E**) In the PET study at 14 weeks, an axial image of the monkey brain receiving grafts into the bilateral putamen showed an increased uptake of ^{18}F-fluorodopa on both sides (R > L), suggesting that the transplanted cells are functionally active. **F**) Immunohistochemistry reveals that grafted cells (Green: BrdU-positive cells incorporated prior to transplantation) survived in the brain and differentiated into DA neurons (Red: TH). Arrows indicate some of the BrdU/TH-positive cells in which nuclei are stained in green, and the cytoplasm and neurites in red, demonstrating that these cells are ES cell-derived DA neurons.

BrdU: Bromodeoxyuridine; DA: Dopaminergic; ES: Embryonic stem; HPLC: High performance liquid chromatography; MPTP: 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PET: Positron emission tomography; SDIA: Stromal cell-derived inducing activity; TH: Tyrosine hydroxylase.

inhibition of apoptosis should improve the cell survival rate. Indeed, Schierle *et al.* reported that treatment of a rat nigral cell suspension with the caspase inhibitor acetyl-tyrosinyl-valyl-alanyl-aspartyl-chloro-methylketone (Ac-YVAD-cmk) prior to transplantation effectively blocked DNA fragmentation (apoptosis), resulting in both a 16-fold decrease in cell death at the acute stage and a 3-fold increase in surviving DA neurons 6 weeks after transplantation [50].

It remains controversial whether immunosuppression is indispensable or not, as allografts of fetal DA neurons have not been rejected without immunosupression in some reports [5,17]. However, rejection by the host immune system (if any) may also prevent effective transplantation. Persistent administration of immunosuppressive drugs is likely to compromise the quality of patients' lives, as suggested by cases of organ transplantation. To bypass this problem, both mouse and human ES cell lines have been generated from somatic cells by nuclear transfer (nt ES cells), enabling us to realise virtually autologous transplantation without immunosuppressive drugs [51,52]. Efficient differentiation into DA neurons

from mouse nt ES cells has already been confirmed [16]. However, at present it is not known whether nt ES cells are safe and whether DA neurons differentiated from nt ES cells have the same lifespan as those from conventional ES cells (the lifespan of the cell is probably programmed in the nucleus). Furthermore, the establishment of nt ES cells involves ethical problems, as discussed below.

5. Ethical problems related to clinical application of human embryonic stem cells

It is crucial to take note of the ethical problems that arise from using human ES cells for research and clinical purposes. Human nt ES cell lines give rise to specific cell types *in vitro* and are available to be used for reconstruction of damaged tissues, a process referred to as 'therapeutic cloning'. However, the generation of cloned human beings by transferring nt ES cells to the uterus ('reproductive cloning') is strictly prohibited. As the initial step of both techniques is identical, it is essential to clarify and establish laws regulating reproductive cloning prior to the establishment of new human ES cell lines by nuclear transfer [53].

Moreover, the destruction of preimplantation blastocysts is necessary to establish human ES cell lines. Attitudes towards the use of human ES cells differ from one country to another based on the religious and cultural backgrounds [54]. For example, in the Catholic world the destruction of preimplantation blastocysts is regarded as abortion, which is prohibited. As a result, laboratory experiments using human ES cells are completely prohibited by law in countries with a strong Catholic influence, such as Ireland, France, Germany, Austria and Italy. On the other hand, the law is less strict in Protestant-influenced countries (UK, Belgium) and allows for the establishment of human ES cell lines by nuclear transfer. Thus, to make reproductive therapy using pluripotent cell lines acceptable to the public in Catholic countries, alternative cell sources will be required. In addition to the great efforts being made to take advantage of pluripotent stem cells located in adult bone marrow [55,56], Kanatsu-Shinohara *et al.* recently reported the successful establishment of an ES cell-like pluripotent cell line from postnatal mouse testis [57]. The potential advantage of these cells is obvious; not only can these cells be obtained without sacrificing embryos, and therefore not raise as many ethical concerns, but they may also represent a potential source of histocompatible tissue for autologous transplantation and may eliminate concerns about tissue rejection that plague ES cell therapy. Nonetheless, even if an alternative cell source proves more amenable to cell replacement therapy than ES cells, the body of knowledge generated by ES cell research will certainly contribute greatly to progress in cell transplantation therapy.

6. Expert opinion and conclusion

In summary, ES cell transplantation therapy for PD should be feasible if methods are found to establish and expand ES cells under defined conditions. So far, a variety of differentiation methods to induce DA neurons from ES cells have been tried, and these are still evolving. The methods to prevent tumour formation by purifying DA neurons, to overcome immunological obstacles by therapeutic cloning or other means, are also under development.

Until now, a great deal of work has focused on donor cells and graft methods; however, future research should next focus on host factors, as creating a permissive environment for the graft to integrate into the host brain is probably of equal importance to supplying the ideal graft. Some evidence suggests that ageing [58-60], inflammation [61] and molecular signals inducing the graft into glial cell fate [62] are involved in both the reduced tissue regenerative potential and inefficient graft integration. Research investigating the molecular mechanisms of these phenomena will provide new insights that will contribute to the success of cell transplantation therapies [61-64].

As a result of great efforts over the last decade, stem cell research may now be poised to bring what was heretofore considered impossible into the realm of reality. We hope that continued work in the future will make cell transplantation therapy for PD as commonplace a procedure as bone marrow transplantation, a currently successful example of cell transplantation therapy.

Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. OLANOW CW, TATTON WG: Etiology and pathogenesis of Parkinson's disease. *Annu. Rev. Neurosci.* (2000) **22**:123-144.
2. BERGMAN H, DEUSCHL G: Pathophysiolosy of Parkinson' disease: from clinical neurology to basic neuroscience and back. *Mov. Disord.* (2002) **17**(Suppl. 3):S28-S40.
3. MIYASAKI JM, MARTIN W, SUCHOWERSKY O, WEINER WJ, LANG AE: Practice parameter: initiation of treatment for Parkinson's disease: an evidence-based review: report of the Quality Standards Subcommittee of the American Academy of Neurology. *Neurology* (2002) **58**:11-17.
4. DOSTROVSKY JO, HUTCHINSON WD, LOZANO AM: The globus pallidus, deep brain stimulation, and Parkinson's disease. *Neuroscientist* (2002) **8**:284-290.
5. FREED CR, GREENE PE, BREEZE RE *et al.*: Transplantation of embryonic dopamine neurons for severe Parkinson's disease. *N. Engl. J. Med.* (2001) **344**:710-719.

•• **Double-blind controlled trial of the transplantation of human embryonic dopamine neurons.**

6. HAGELL P, BRUNDIN P: Cell survival and clinical outcome following intrastriatal transplantation in Parkinson's disease. *J. Neuropathol. Exp. Neurol.* (2001) **60**:741-752.

7. LINDVALL O, SAWLE G, WIDNER H *et al.*: Evidence for long-term survival and function of dopaminergic grafts in progressive Parkinson's disease. *Ann. Neurol.* (1994) **35**:172-180.
8. OLANOW CW, GOETZ CG, KORDOWER JH *et al.*: A double blind controlled trial of bilateral fetal nigral transplantation in Parkinson's disease. *Ann. Neurol.* (2003) **54**:403-414.
9. WIDNER H, TETRUD J, REHNCRONA S *et al.*: Bilateral fetal mesencephalic grafting in two patients with parkinsonism induced by 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP). *N. Engl. J. Med.* (1992) **327**:1556-1563.
10. SMITH AG: Embryo-derived stem cells: of mice and men. *Annu. Rev. Cell Dev. Biol.* (2001) **17**:435-462.
11. DONOVAN PJ, GEARHART J: The end of the beginning for pluripotent stem cells. *Nature* (2001) **414**:92-97.
12. LEE SH, LUMELSKY N, STUDER L, AUERBACH JM, MCKAY RD: Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cell. *Nat. Biotech.* (2000) **18**:675-679.
- **First evidence for the generation of DA neurons from mouse ES cells.**
13. KAWASAKI H, MIZUSEKI K, NISHIKAWA S *et al.*: Induction of midbrain dopaminergic neurons from ES cells by stromal cell-derived activity. *Neuron* (2000) **28**:31-40.
14. BJÖRKLUND LM, SANCHEZ-PERNAUTE R, CHUNG S *et al.*: Embryonic stem cells develop into functional dopaminergic neurons after transplantation in a Parkinson rat model. *Proc. Natl. Acad. Sci. USA* (2002) **99**:2344-2349.
15. KIM JH, AUERBACH JM, RODRIGUEZ-GOMEZ JA *et al.*: Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. *Nature* (2002) **418**:50-56.
- **One of a series of papers demonstrating functional improvement by transplantation of mouse ES cell-derived DA neurons.**
16. BARBERI T, KLIVENYI P, CALIGASAN NY *et al.*: Neural subtype specification of fertilization and nuclear transfer embryonic stem cells and application in parkinsonian mice. *Nat. Biotechnol.* (2003) **10**:1200-1207.
17. STUDER L, TABAR V, MCKAY RD: Transplantation of expanded mesencephalic precursors leads to recovery in parkinsonian rats. *Nat. Neurosci.* (1998) **1**:290-295.
18. EVANS MJ, KAUFMAN M: Establishment in culture of pluripotential cells from mouse embryos. *Nature* (1981) **292**:154-156.
- **Establishment of mouse ES cells.**
19. THOMSON JA, ITSKOVITZ-ELDOR J, SHAPIRO SS *et al.*: Embryonic stem cell lines derived from human blastocysts. *Science* (1998) **282**:1145-1147.
- **Establishment of human ES cells.**
20. SMITH AG, HEATH JK, DONALDSON DD *et al.*: Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* (1998) **336**:688-690.
- **Mouse ES cells can be maintained under feeder cell-free conditions.**
21. SATO N, MEIJER L, SKALTSOUNIS L, GREENGARD P, BRIVANLOU AH: Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nat. Med.* (2004) **10**:55-63.
22. YING QL, NICHOLS J, CHAMBERS I, SMITH A: BMP induction of Id proteins suppresses differentiation and sustains embryonic stem cell self-renewal in collaboration with STAT3. *Cell* (2003) **115**:281-292.
23. NAKASHIMA K, TAKIZAWA T, OCHIAI W *et al.*: BMP2-mediated alteration in the developmental pathway of fetal mouse brain cells from neurogenesis to astrocytogenesis. *Proc. Natl. Acad. Sci. USA* (2001) **98**:5868-5873.
24. HOLLNAGEL A, OEHLMANN V, HEYMER J, RUTHER U, NORDHEIM AJ: *Id* genes are direct targets of bone morphogenetic protein induction in embryonic stem cells. *J. Biol. Chem.* (1999) **274**:19838-19845.
25. LOEBEL DA, WATSON CM, DE YOUNG RA, TAM PP: Lineage choice and differentiation in mouse embryos and embryonic stem cells. *Dev. Biol.* (2003) **264**:1-14.
26. MARTIN GR: Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. USA* (1981) **78**:7634-7638.
27. BAIN G, KITCHENS D, YAO M, HUETTNER JE, GOTTLIEB DI: Embryonic stem cells express neuronal properties *in vitro*. *Dev. Biol.* (1995) **168**:342-357.
28. WICHTERLE H, LIEBERAM I, PORTER JA, JESSELL TM: Directed differentiation of embryonic stem cells into motor neurons. *Cell* (2002) **110**:385-397.
29. MIZUSEKI K, SAKAMOTO T, WATANABE K *et al.*: Generation of neural crest-derived peripheral neurons and floor plate cells from mouse and primate embryonic stem cells. *Proc. Natl. Acad. Sci. USA* (2003) **100**:5828-5833.
30. KUO HC, PAU KY, YEOMAN RR, MITALIPOV SM, OKANO H, WOLF DP: Differentiation of monkey embryonic stem cells into neural lineages. *Biol. Reprod.* (2003) **68**:1727-1735.
31. KAWASAKI H, SUEMORI H, MIZUSEKI K *et al.*: Generation of dopaminergic neurons and pigmented epithelia from primate ES cells by stromal cell-derived inducing activity. *Proc. Natl. Acad. Sci. USA* (2002) **99**:1580-1585.
- **First evidence for the generation of DA neurons from primate ES cells.**
32. PERRIER AL, TABAR V, BARBERI T *et al.*: Derivation of midbrain dopamine neurons from human embryonic stem cells. *Proc. Natl. Acad. Sci. USA* (2004) **101**:12543-12548.
- **First evidence for the generation of DA neurons from human ES cells.**
33. BUYTAERT-HOEFEN KA, ALVAREZ E, FREED CR: Generation of tyrosine hydroxylase positive neurons from human embryonic stem cells after coculture with cellular substrates and exposure to GDNF. *Stem Cells* (2004) **22**:669-674.
34. ZENG X, CAI J, CHEN J *et al.*: Dopaminergic differentiation of human embryonic stem cells. *Stem Cells* (2004) **22**:925-940.
35. TROPEPE V, HITOSHI S, SIRARD C, MAK TW, ROSSANT J, VAN DER KOOY D: Direct neural fate specification from embryonic stem cells: a primitive mammalian neural stem cell stage acquired through a default mechanism. *Neuron* (2001) **30**:65-78.
36. YING QL, STAVRIDIS M, GRIFFITHS D, LI M, SMITH A: Conversion of embryonic stem cells into neuroectodermal precursors in adherent monocultures. *Nat. Biotechnol.* (2003) **21**:183-186.

37. WATANABE K, KAMIYA D, NISHIYAMA A *et al.*: Directed differentiation of telencephalic precursors from embryonic stem cells. *Nat. Neurosci.* (2005) **8**:288-296.

38. SHULZ TC, NOGGLE SA, PALMARINI GM *et al.*: Differentiation of human embryonic stem cells to dopaminergic neurons in serum-free suspension culture. *Stem Cells* (2004) **22**:1218-1238.

39. YAN Y, YANG D, ZARNOWSKA ED *et al.*: Directed differentiation of dopaminergic neuronal subtypes from human embryonic stem cells. *Stem Cells* (2005) **23**:781-790.

40. TAKAGI Y, TAKAHASHI J, SAIKI H *et al.*: Dopaminergic neurons generated from monkey embryonic stem cells function in a Parkinson primate model. *J. Clin. Invest.* (2005) **115**:102-109.

•• **First evidence demonstrating functional improvement by transplantation of primate ES cell-derived DA neurons into lesioned monkeys.**

41. OHMACHI S, WATANABE Y, MIKAMI T *et al.*: FGF-20, a novel neurotrophic factor, preferentially expressed in the substantia nigra pars compacta of rat brain. *Biochem. Biophys. Res. Commun.* (2000) **277**:355-360.

42. BURNS RS, CHIUEH CC, MARKEY SP, EBERT MH, JACOBOWITZ DM, KOPIN IJ: A primate model of parkinsonism: selective destruction of dopaminergic neurons in the pars compacta of the substantia nigra by N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Proc. Natl. Acad. Sci. USA* (1983) **80**:4546-4550.

43. ERDÖ F, BUHRLE C, BLUNK J *et al.*: Host-dependent tumorigenesis of embryonic stem cell transplantation in experimental stroke. *J. Cereb. Blood Flow Metab.* (2003) **23**:780-785.

44. REUBINOFF BE, ITSYKSON P, TURETSKY T *et al.*: Neural progenitors from human embryonic stem cells. *Nat. Biotechnol.* (2001) **19**:1134-1140.

45. ZHANG SC, WERNIG M, DUNCAN ID, BRUSTLE O, THOMSON JA: *In vitro* differentiation of transplantable neural precursors from human embryonic stem cells. *Nat. Biotechnol.* (2001) **19**:1129-1133.

46. BEN-HUR T, IDELSON M, KHANER H *et al.*: Transplantation of human embryonic stem cell-derived neural progenitors improves behavioral deficit in Parkinsonian rats. *Stem Cells* (2004) **22**:1246-1255.

47. NISHIMURA F, NOSHIKAWA M, KANDA S *et al.*: Potential use of embryonic stem cells for the treatment of mouse parkinsonian models: improved behavior by transplantation of *in vitro* differentiated dopaminergic neurons from embryonic stem cells. *Stem Cells* (2003) **21**:171-180.

48. WOOD HB, EPISKOPOU V: Comparative expression of the mouse Sox1, Sox2 and Sox3 genes from pre-gastrulation to early somite stages. *Mech. Dev.* (1999) **86**:197-201.

49. BIEBERICH E, SILVA J, WANG G, KRISHNAMURTHY K, CONDIE BG: Selective apoptosis of pluripotent mouse and human stem cells by novel ceramide analogues prevents teratoma formation and enriches for neural precursors in ES cell-derived neural transplants. *J. Cell Biol.* (2004) **167**:723-734.

50. SCHIERLE GS, HANSSON O, LEIST M, NICOTERA P, WIDNER H, BRUNDIN P: Caspase inhibition reduces apoptosis and increases survival of nigral transplants. *Nat. Med.* (1999) **5**:97-100.

51. WAKAYAMA T, TABAR V, RODRIGUEZ I, PERRY AC, STUDER L, MOMBAERTS P: Differentiation of embryonic stem cell lines generated from adult somatic cells by nuclear transfer. *Science* (2001) **292**:740-743.

• **First evidence for the generation of mouse nt ES cells.**

52. HWANG WS, RYU YJ, PARK JH *et al.*: Evidence of a pluripotent human embryonic stem cell line derived from a cloned blastocyst. *Science* (2004) **303**:1669-1674.

•• **First evidence for the generation of human nt ES cells.**

53. FISCHBACH GD, FISCHBACH RL: Stem cells: science, policy, and ethics. *J. Clin. Invest.* (2004) **114**:1364-1370.

54. KNOWLES LP: A regulatory patchwork-human ES cell research oversight. *Nat. Biotechnol.* (2004) **22**:157-163.

55. JIANG Y, JAHAGIRDAR BN, REINHARDT RL *et al.*: Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* (2002) **418**:41-49.

• **Identification of pluripotent stem cells in adult tissues.**

56. DEZAWA M, KANNO H, HOSHINO M *et al.*: Specific induction of neuronal cells from bone marrow stromal cells and application for autologous transplantation. *J. Clin. Invest.* (2004) **113**:1701-1710.

57. KANATSU-SHINOHARA M, INOUE K, LEE J *et al.*: Generation of pluripotent stem cells from neonatal mouse testis. *Cell* (2004) **119**:1001-1012.

• **First evidence for the generation of pluripotent stem cells from postnatal gonads.**

58. CONBOY IM, CONBOY MJ, SMYTHE GM, RANDO TA: Notch-mediated restoration of regenerative potential to aged muscle. *Science* (2003) **302**:1575-1577.

59. MORRISON SJ, WANDYCZ AM, AKASHI K, GLOBERSON A, WEISSMAN II: The aging of hematopoietic stem cells. *Nat. Med.* (1996) **2**:1011-1016.

60. KUHN HG, DICKSON-ANSON H, GAGE FH: Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. *J. Neurosci.* (1996) **16**:2027-2033.

61. MONJE L, TODA H, PALMER TD: Inflammatory blockade restores adult hippocampal neurogenesis. *Science* (2003) **302**:1760-1765.

62. LIM DA, TRAMONTIN AD, TREVEJO JM, HERRERA DG, GARCIA-VERDUGO JM, ALVAREZ-BUYLLA A: Noggin antagonizes BMP signaling to create a niche for adult neurogenesis. *Neuron* (2000) **28**:713-726.

63. IAKOVA P, AWAD SS, TIMCHENKO NA: Aging reduces proliferative capacities of liver by switching pathways of C/EBPe growth arrest. *Cell* (2003) **113**:495-506.

64. CONBOY IM, CONVOY MJ, WAGERS AJ, GIRMA ER, WEISSMAN IL, RANDO TA: Rejuvenation of aged progenitor cells by exposure to a young systemic environment. *Nature* (2005) **433**:760-764.

• **Age-related decline of tissue regenerative potential is compensated by unknown factors contained in the serum of young animals.**

Affiliation

Hitoshi Fukuda[1] & Jun Takahashi[†1] MD, PhD
[†]Author for correspondence
[†1]Kyoto University Graduate School of Medicine, Department of Neurosurgery, 54 Kawahara-cho, Shogoin, Sakyo, Kyoto 606-8507, Japan
Tel: +81 75 751 3450; Fax: +81 75 752 9501; E-mail: jbtaka@kuhp.kyoto-u.ac.jp