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CHAPTER 14

# Neural grafting in Parkinson's disease: problems and possibilities

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**Abstract:** Neural transplantation has emerged as a possible therapy for Parkinson's disease (PD). Clinical studies performed during the 1990s, where dopaminergic neurons derived from the human embryonic brain were transplanted into striatum of patients with PD, provided proof-of-principle that long-lasting therapeutic benefits can be achieved. Subsequent studies, in particular two that followed a double-blind, sham surgery, placebo-control design, showed variable and mostly negative results. They also revealed that some patients develop involuntary movements, so called graft-induced dyskinesias, as side effects. Thus, while nigral transplants clearly work well in select PD cases, the technique needs refinement before it can successfully be performed in a large series of patients. In this review, we describe the clinical neural transplantation trials in PD and the likely importance of factors such as patient selection, trial design, preparation of the donor tissue, and surgical techniques for successful outcome and avoiding unwanted side effects. We also highlight that it was recently found that neuropathological signs typical for PD can appear inside some of the grafted neurons over a decade after surgery. Finally, we discuss future possibilities offered by stem cells as potential sources of dopamine neurons that can be used for transplantation in PD.

**Keywords:** Parkinson's disease; Dopamine neuron; Transplantation; Dyskinesias; Lewy bodies; Stem cells

## Introduction

Over the past 30 years, neural transplantation has emerged as a possible therapy for Parkinson's disease (PD). Today we know that grafted neural cells can survive for over 20 years and exert beneficial effects in PD patients. Results obtained during the 1990s in open-label trials with grafted dopaminergic neurons derived from the human embryonic brain were very encouraging. The patients displayed impressive improvements of symptoms and restoration of dopaminergic

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neurotransmission. By contrast, two double-blind, sham surgery, placebo-controlled trials with nigral transplants in PD reported no improvement in grafted groups on primary endpoints. These trials also highlighted that some patients develop involuntary movements, so called graft-induced dyskinesias (GIDs), as side effects. Thus, while nigral transplants clearly work well in select PD cases, the technique needs refinement and is difficult to successfully perform in a large series of patients.

The aims of this review are to briefly review clinical neural transplantation trials in PD and describe factors that may influence likelihood of a successful outcome, such as patient selection, transplantation technique, and trial design. We underscore the problem of GIDs and how they might be avoided in the future. We describe other practical obstacles linked to fetal tissue transplantation that currently prevent transfer of the technology into an established treatment, and how they might be circumvented in a forthcoming multicenter trial that is sponsored by the European Commission. We highlight recent findings that neuropathological features typical for PD appear inside the grafted neurons over a decade after surgery. Finally, we discuss future possibilities offered by stem cells as potential sources of dopamine neurons that can be used for transplantation in PD.

## Open-label transplantation trials in PD

Clinical trials using fetal ventral mesencephalic (VM) tissue began in the late 1980s in Mexico (Madrazo et al., 1988) and Sweden (Lindvall et al., 1989) with tissue from 12–14–week- and 6–8-week-old fetuses/embryos, respectively. The tissue was transplanted into the striatum with minimal clinical improvement, at least in the patients from Sweden. However, by refining the techniques, several subsequent open-label clinical studies demonstrated that the patients can display significant improvements following implantation of fetal dopamine neurons. These trials took place in different centres across Europe and the US and involved mainly patients with idiopathic PD, although three (two of whom which have been reported in the literature) patients with MPTP-induced parkinsonism were also grafted using this approach (Brundin et al., 2000; Freed et al., 1992; Hauser et al., 1999; Lindvall et al., 1990, 1994; Peschanski et al., 1994; Spencer et al., 1992; Wenning et al., 1997; Widner et al., 1992). Whilst many patients benefited from the procedure, not everyone improved. The patients fell into three main categories: those exhibiting marked benefit of a clear therapeutic value and in some cases permitting withdrawal of anti-parkinsonian medication; those showing significant improvements, but to a modest degree and still needing continued medication; and finally, those who never displayed any measureable benefit. Nevertheless, even in those where marked effects were seen, the benefits were confined to certain aspects of motor control including improvements in the motor aspects of the Unified Parkinson's Disease Rating Scale (UPDRS) in the defined off period as well as a range of timed motor tasks. This in turn had a positive impact on Activities of Daily Living (ADL) and health-related quality of life with reduced L-dopa requirements (Freed et al., 1992; Hauser et al., 1999; Lindvall et al., 1990, 1994; Peschanski et al., 1994; Spencer et al., 1992; Wenning et al., 1997; Widner et al., 1992). Importantly though, not all motor features of PD improved, in particular, features such as tremor and postural instability improved least, as one might expect given that these features of disease generally respond poorly to L-dopa therapy. Furthermore, non-motor features of PD were not investigated to any significant extent, mainly because the condition was seen as being primarily a movement disorder in the 1980s–1990s, with few if any non-motor features. This understanding of PD has, of course, been radically revised in recent years as the condition is now known to encompass as many non-motor as motor features (Foltynie et al., 2002; Lewis et al., 2005; Williams-Gray et al., 2009).

The clinical improvements reported in these early open-label neural grafting trials have been, in many cases, long-lasting and associated with evidence of surviving dopaminergic cells within the grafts. The evidence for this has come from two main sources; firstly through functional imaging in which it has been shown that the grafts are associated with increased fluorodopa (F-dopa) uptake on positron emission tomography (PET) scans, as well as regulated dopamine release and re-activation of motor cortical areas (Piccini et al., 1999, 2000). Secondly, post-mortem studies have been undertaken in patients that have died after grafting from unrelated causes. In these patients, there is evidence for the survival of grafted fetal VM dopaminergic neurons with local reinnervation of the striatum by these cells (Hauser et al., 1999; Kordower et al., 1996). Such studies have suggested that around 100,000 dopaminergic neurons need to be present within the grafted striatum to achieve significant clinical benefit (Hagell and Brundin, 2001), and that those of nigral origin (as opposed to non-nigral dopaminergic midbrain neurons) are most able to innervate the striatum.

These initial open-label clinical studies continued through the 1990s and were very important in showing that fetal VM allografts (transplants between genetically dissimilar individuals within the same species) could survive in patients with advanced PD, become functionally integrated, and produce sustained clinical benefits. However, it also soon became clear that transplants of this type produced very variable responses, with some patients showing only little improvement or transient benefits. The reason for this was not immediately clear, in that it was uncertain whether it was a technical issue to do with the tissue preparation and implantation procedure or a more fundamental issue to do with patient selection and stage of disease (Bjorklund et al., 2003; Hagell and Brundin, 2001).

Whilst this debate was being undertaken to try and explain this variability and how it could be minimized, the results from two double-blind, placebo-controlled trials of fetal VM transplantation in PD were published which brought the issue centerstage (Freed et al., 2001; Olanow et al., 2003). This issue still causes heated debate today, especially as newer more effective therapies for the motor features of advanced PD have become available such as deep brain stimulation (DBS) and apomorphine/Duodopa® infusion therapies (Lewis et al., 2003).

## Double-blind placebo-controlled transplantation trials in PD

As the 1990s progressed, more data emerged from a number of open-label VM transplant studies in PD showing that these transplants were effective at treating advanced PD in some cases, although the emerging use of DBS caused some to question whether this was a better, more practical, solution in the management of advanced motor PD. However, with a change in administration in the US and with encouragement from the initial open-label results, two double-blind placebo-controlled trials of fetal VM transplantation in PD were funded by the National Institutes of Health (NIH), USA. These studies sought to show that VM grafting in patients with PD was able to produce a real effect over and above any placebo-mediated benefits and to show that this technique was really working through a dopaminergic reinnervation mechanism and not some non-specific effect as had been the case in the 1980s with adrenal medullary transplants (Barker and Dunnett, 1993).

The first of these controlled trials was published in 2001 by Freed et al. and involved 40 PD patients aged between 34 and 75 years (half under the age of 60 and half over) and with advanced disease (mean disease duration of 14 years) (Freed et al., 2001). Patients were randomly assigned to receive a transplant or imitation, sham surgery. The latter involved patients going to theatre and having a burr hole but with no penetration of the dura, such that the patient and subsequently the assessing neurologist did not know whether

the individual truly had a transplant or just imitation surgery. Finally, 33 patients (some from the initial control group, after unblinding of the study) ended up receiving grafts (Ma et al., 2010).

The VM tissue was obtained from aborted 7–8-week-old embryos cultured in F12 medium containing 5% human placental serum for 1–4 weeks prior to transplantation and unusually (compared to the other transplant trials) prepared into strands or noodles of tissue (Clarkson et al., 1998).

The transplants took place under local anesthetic with the patient awake and tissue from two embryos was transplanted into the putamen on each side of the patient's brain. Immunosuppression was not given, unlike the open-label studies that had all used standard immunotherapy (Freed et al., 2001).

The primary outcome was a change in a subjective global rating of clinical improvement at 1 year post transplant. This revealed that there was no significant improvement in the transplanted patients compared to the sham surgery group (Freed et al., 2001). In further analysis, significant improvements were seen using more traditional measures such as the UPDRS motor scores in defined "off" times in grafted patients who were less than 60 years of age. Subsequent analysis suggested, however, that the main determinant of this correlation was the preoperative L-dopa responsiveness rather than the age of the patient, as even older patients with good preoperative L-dopa responsiveness showed improvement. In a recent report describing the long-term outcome (up to 4 years) in some of the patients from the same cohort, it now appears that a high residual preoperative level of dopamine in the anterior putamen, as determined by F-dopa PET, was associated with better clinical outcome (Ma et al., 2010). Interestingly, the early claims that younger patients respond better to grafting do not hold up. The conclusion is that the older patients simply have a slower rate of recovery and eventually catch up with the younger ones (Ma et al., 2010).

The main motor improvements were similar to those seen with dopaminergic medications and included rigidity and in the younger patients, bradykinesia, while tremor showed no response. Despite the modest and variable clinical benefits, PET scanning showed significant increases in F-dopa uptake in the putamen of the transplant group compared to placebo and post-mortem examinations showed dopaminergic neuronal survival and fiber outgrowth in the grafts. However, the number of surviving grafted dopamine neurons was less than that reported in patients showing greater response in open-label studies (e.g., ~11,600–20,200 per graft at 7 months and ~2100–22,800 at 3 years) (Freed et al., 2001).

This failure to show benefit in the primary outcome parameter was disappointing. In itself this would have been discouraging, but of greater concern in this trial was the first clear description of the development of significant GIDs in 15% of the transplanted patients more than 1 year post transplant—dyskinesias that occurred in the absence of medication, but presence of the graft. Several of these patients required further surgical intervention with subthalamic DBS to relieve them of these GIDs (Olanow et al., 2001).

The reason for these trials failing to achieve their primary outcomes coupled to the development of GIDs is still debated and obviously is in need of resolution in order for the field to move forward. However in the first of the two NIH-sponsored studies (Freed et al., 2001), a number of critical factors have been identified. In particular the following issues were problematic:

- The primary endpoint was a subjective one. The patients mailed in to the clinic their impressions of how their clinical state had changed compared to 1 year earlier. This could give misleading data by virtue of the fact that patients expectations may be greater than that which a transplant can deliver at 1 year, and patients do not always perceive improvements in their PD as can be seen when medication is stopped as they feel the tablets are not working, only for them to get much worse.
- The follow-up time was only 1 year, which is short especially considering that the grafted

neurons are embryonic at the time of implantation. In some of the open-label studies the maximal benefit from the transplant was not seen until 3 years after grafting. Interestingly, the recent follow-up report of a subgroup from the same patient cohort showed that they continued to improve in the UPDRS motor score in "defined off" between 1 and 2 years after grafting (Ma et al., 2010). Indeed, there was a sustained 25% improvement in UPDRS motor score in the "defined off" at 4 years with a 45% increase in F-dopa signal in the grafted putamen over the same period of time. This suggests that a longer follow-up might have led to significant graft-induced improvement being detected also in the subjective global rating scale.

- The amount of tissue used, in terms of number of donor fetuses, was less (two per putamen) than some other studies.
- The preparation of the tissue involved prolonged incubation times which could have adversely affected the survival of the dopaminergic neurons, as suggested by the post-mortem findings in this study (Freed et al., 2001).
- The absence of immunosuppression may have further compromised the viability of the dopaminergic cells in the transplants.
- The neurosurgical approach was transfrontal with the tissue being placed as two long noodles into each putamen, which may have contributed to the development of dopaminergic hotspots in the grafted striatum (Ma et al., 2002).
- The placebo arm of the trial was offered a transplant after a year and 13 out of the 20 patients were therefore subsequently grafted and by so doing the comparator control arm of the trial was lost.

All of this may help explain why patients did not show significant improvements in their clinical state at the primary outcome parameter, and also why the transplants contained fewer dopaminergic cells at post-mortem compared to other histopathological studies. As mentioned above, the two cases reported in the original paper contained only 6800–38,400 grafted dopamine neurons per putamen (Freed et al., 2001). In addition, a follow-up PET study by Ma et al. showed dopaminergic hotspots, especially in patients developing GID (Ma et al., 2002). Although these hotspots were not described in the PET results from longer follow-up times (Ma et al., 2010), the mode of cell preparation and graft implantation procedure may have contributed to the striatal complex initially being innervated unevenly by the transplants.

For all these reasons, it could be argued that the first controlled neural grafting trial in PD produced negative results with side effects for methodological reasons, rather than from a fundamental problem with VM transplants for PD *per se*. This concern, i.e., that the neural transplantation technique in PD was not sufficiently well developed to merit a placebo-controlled trial, had been raised by European investigators already before the trial was initiated (Widner, 1994). In 2003, however, a second controlled study was published with a negative outcome that suggested that the original trial result could not be dismissed as a methodological aberration.

This second NIH-sponsored study of Olanow et al. (2003) involved 34 patients with advanced PD, aged between 30 and 75 years. Patients were again randomized to receive either bilateral transplants or sham surgery, but in this study patients were transplanted with either one or four donors in each putamen with a different surgical technique and using immunosuppression. Solid pieces of VM tissue were obtained from 6–9-week fetuses, stored in a hibernation medium for 2 days prior to transplantation. The same group had used an identical tissue dissection and preparation protocol successfully in earlier open-label studies (Hauser et al., 1999). The surgery was performed under general anesthesia using a two-stage procedure separated by a week, with the tissue from one or four embryos being transplanted into the putamen bilaterally. All patients received immunosuppression with cyclosporine monotherapy that was maintained for 6 months postoperatively (Olanow et al., 2003). As in the Freed et al. study, sham

surgery consisted of partial burr holes only with no breach of the dura.

In this trial, the primary outcome measure was a very standard one and involved a change in the motor component of the UPDRS in the practically defined "off" state, between the baseline and the final 24-month visit. Once again, no significant overall treatment effect was observed, although there was a clear trend for benefit as one moved from sham-operated patients to those receiving tissue from one and four embryos. However, this was only a trend and not statistically significant. Notably, the group sizes were small at 11–12 in each therapeutic arm. There were no changes in any of the secondary motor measures. Furthermore, the grafts caused significant motor benefits if the patients were divided by disease stage, with less advanced patients doing significantly better post grafting (Olanow et al., 2003). Importantly, patients in both the one- and four-donor transplant groups showed significant motor improvement compared to placebo at 6 and 9 months post transplant, but not thereafter. The apparent loss of benefit partially coincided with the discontinuation of their immunosuppressive therapy. Thus, it is possible that this triggered an immune response to the graft, compromising the function of the grafted dopaminergic neurons and contributing to an apparent loss of transplant function at 2 years. Indeed the magnitude and time course of the initial improvement (up to 6–9 months) was similar to that reported for previous open-label studies (Brundin et al., 2000; Freeman et al., 1995; Lindvall et al., 1990), which further supports this interpretation of the data. Despite a possible immune system-mediated impairment in graft function, PET scanning showed significant bilateral increases in striatal F-dopa uptake in both transplant groups, with the four-donor group showing the greatest increase. This fitted well with post-mortem data from this study showing that the dopaminergic neurons survived in large numbers (around 100,000 per putamen in the four-donor group and 30,000 in the one-donor group) with marked reinnervation of the striatum (Olanow et al., 2003).

The results of the study by Olanow and coworkers would have encouraged many in the field to see this therapy as having potential, but as in the Freed et al. study the development of significant "off-medication" GIDs in 56.5% of the grafted patients 6–12 months after transplantation generated doubts about the safety of the surgery (Olanow et al., 2003). These GIDs typically consisted of stereotypic, rhythmic movements of one or both lower extremities, with three patients requiring further surgical intervention to reduce their severity.

Thus, the results from these two double-blind placebo-controlled trials raised serious concerns about the utility and safety of fetal VM transplants in patients with PD. An anxiety that was magnified by the relative safety and efficacy of DBS surgery in advanced PD and the change in presidential administration in the US in 2001 to one that discouraged research involving human fetal tissue.

## How does one reconcile differences in outcomes of open-label and double-blind trials?

There are a number of possible interpretations. Firstly, one could take a very dogmatic approach, concluding that open-label studies are subject to patient and assessor bias with placebo effect and thus the double-blind studies reveal the true answer—namely neural grafting does "not" really work in PD.

Secondly, one could take the opposite view and say that the double-blind studies were inadequately powered to see any benefit. Thus, they might be subject to Type II errors, namely the studies are so powered that there is a high risk that no difference will be seen when in reality there is one, and as such the only useful data we have is from individual patients and their responses in both the open-label and double-blind studies that suggest that neural grafting in PD works.

A third interpretation would be that all trials used suboptimal donor tissue dissections, tissue preparation methods, and surgical techniques.

Thus, the data so obtained tells us nothing significant about the true potential of neural transplantation in PD, and until the techniques are better developed no further trials should be done.

A fourth and final way of approaching the data is to adopt a position of greater equipoise and say that all trials to date have their problems, but buried within the trial data are clues that this approach can work in some cases. Defining what is special about those cases would then enable VM transplants to be used in some PD patients again, albeit in a modified form.

Today most people actively working with neural transplantation research would subscribe to this latter view. They accept that ultimately if cell transplantation is going to be useful and competitive in PD then it will need to be subject to a properly powered double-blind study, which may not involve sham surgery but best alternative therapy. If we accept this, then are there any clues from the studies to date as to what may be the critical methodological and neurobiological factors that govern whether PD patients will respond well to neural grafts.

Perhaps the easiest way to identify the critical factors, short of a meta-analysis of all the trials which is not currently possible, is to look specifically at issues of patient selection, graft tissue preparation and placement; the extent of immunosuppression; GIDs; and trial design. We will now deal with each of these items in turn.

### *Patient selection*

It is now recognized that PD is not a single homogenous disorder, even if one excludes cases of Mendelian forms of Parkinsonism. Our own work has clearly defined patients with different cognitive profiles and disease courses (Foltynie et al., 2002; Lewis et al., 2005; Williams-Gray et al., 2009). It is highly likely that the transplant trials to date have been contaminated by this, and some of the grafted patients may not even have suffered from PD, given the diagnostic difficulties that exist with PD (Hughes et al., 2002). Furthermore, it is not only the "type" of PD that is important in predicting response to grafting, but also where in the course of the disease they lie. So, for example, the best results in the double-blind placebo-controlled trials were seen in patients with less severe disease (UPDRS < 49 at baseline), best preoperative response to L-dopa (Bjorklund et al., 2003), and least loss of dopamine in the anterior putamen (Ma et al., 2010). Older patients may have responded equally well, but the rate of improvement was slower (Ma et al., 2010). In addition, other open-label studies have suggested that preoperative sparing in the ventral putamen is an important predictor of a good graft response and patients with dopamine loss that extends out of the dorsal striatum might do less well post grafting (Piccini et al., 2005). This may reflect the fact that these patients either have a different type of PD or are at a different, possibly more advanced, stage of disease. Thus choosing younger patients with less advanced disease and dopamine loss restricted to the dorsal and posterior putamen may produce better results with VM grafts.

### *Graft tissue preparation and placement*

As has already been alluded to, the amount of tissue grafted, along with its preparation and mode of implantation, may all be important in graft survival and efficacy. This therefore includes issues of the number and age of embryos used; the way in which this fetal tissue is prepared and stored prior to implantation; and the actual technique used to implant that tissue. So, for example, in the two NIH-funded studies, Freed et al., in comparison to the previous open-label studies, used less tissue, stored for longer times, delivered by a transfrontal approach (Freed et al., 2001), whilst in the Olanow et al. study tissue pieces were implanted after being stored for short periods of time (Olanow et al., 2003). From studies in experimental animals, it is well known that

immature dopaminergic neurons in the embryonic VM are very susceptible to damage and death when subjected to trauma or cell stress. As a result, many factors in the tissue preparation process are known to adversely affect the survival of grafted dopaminergic neurons (Brundin et al., 2000; Laguna Goya et al., 2008). For example, prolonged storage times will adversely affect dopaminergic cell viability in VM grafts (Freeman and Brundin, 2006). Finally even in the highly controlled environment of neural allografting in experimental models of PD, graft variability in terms of dopaminergic cell survival is commonly seen for reasons that are not altogether clear. Thus, it must be assumed that tissue preparation will significantly impact graft survival in patients.

### *Immunosuppression*

A fundamental question relates to whether it is necessary or not to immunosuppress a patient receiving an intracerebral neural allograft. As is well known, the brain is considered to be an immunologically privileged site by virtue of its blood–brain barrier (BBB), the absence of professional antigen-presenting cells and a properly developed lymphatic system (Barker and Widner, 2004; Sayles et al., 2004; Widner and Brundin, 1988). However, in the transplant situation this is significantly compromised as the BBB is breached by the grafting procedure. The trauma of the grafting itself triggers a local inflammatory response, with upregulation of major histocompatibility complex (MHC) antigen expression on the cells within, and around, the fetal implant (Duan et al., 1995). This means that parts of the grafted material might be presented to the immune system locally by astrocytes/microglia acting as antigen-presenting cells and/or antigen from the graft might drain to the deep cervical lymph nodes resulting in a peripherally-induced immune allorejection response. This immune rejection may only be partial and resolve over time, as we know that large VM grafts can survive in patients who have not been immunosuppressed or in whom the immunosuppression was stopped years previously. In line with this, PET imaging does not reveal any changes in graft-mediated F-dopa uptake when immunosuppression is terminated (Piccini et al., 2005). However, a complete failure to give immunosuppression or a too short course of it might significantly compromise survival or function of grafted dopaminergic neurons, even though the graft is not completely rejected. This criticism could be levelled at some of the clinical transplant studies, most notably the two NIH-funded trials. In the Freed et al. study, no immunosuppression was given (Freed et al., 2001). By contrast, in the Olanow et al. trial immunosuppression with cyclosporine was given, albeit only for 6 months, and there was deterioration in clinical response 6–9 months after grafting, with post-mortem tissue evidence for activated microglia and immune reactivity in and around the grafts (Olanow et al., 2003, 2009).

### *Graft-induced dyskinesias (GIDs)*

Perhaps the single most important factor adversely affecting the development of cell-based therapies for PD has been the discovery that GIDs occur in a significant subset of grafted patients. Although mild in many cases, this side-effect has been disabling and in some instances requiring further neurosurgical intervention. Definitely, GIDs need to be better understood if we are to apply neural grafting to new cohorts of patients with PD. It must be recognized that GIDs (Cubo et al., 2001; Hagell et al., 2002) are different in nature to the typical dyskinesias seen in drug-treated PD (Cubo et al., 2001; Luquin et al., 1992).

The reasons for, and the mechanism behind, the development of GID are still not fully understood, and a number of mechanisms have been suggested (Hagell and Cenci, 2005). Both clinical observations and recent studies in rodent models of GID have shed some light on the problem. An important observation is that in all patients where GIDs

have been seen, L-dopa-induced dyskinesias (LIDs) were present preoperatively. However, there was no correlation between the severity of the LIDs and the likelihood of the patient developing GIDs post-operatively (Hagell et al., 2002). Studies in animal models of GID have involved drug administration and not spontaneous GIDs and are therefore not the perfect model of what is seen in the patients. Nevertheless, they have also shown that prior priming with L-dopa-induced abnormal involuntary movements is necessary for the graft-induced abnormal movements to develop later (Lane et al., 2009; Steece-Collier et al., 2009). The relatively inhomogeneous graft-mediated dopaminergic reinnervation of the host putamen (Ma et al., 2002) has also been suggested to promote GID by causing excessive release of dopamine in "hotspots". Studies in grafted rats on amphetamine-induced abnormal involuntary movements have provided some tentative support for the "hotspot" hypothesis in that they have shown that amount of graft-derived innervation and the precise placement of the implant within the striatum influence the abnormal movements (Carlsson et al., 2006; Lane et al., 2006). The observation that GIDs typically develop after a delay in patients without immunosuppression or after immunosuppressive therapy has been discontinued (Freed et al., 2001; Olanow et al., 2003; Piccini et al., 2005), led to the suggestion that they may be due to a local neuroinflammatory response secondary to a low-grade immune rejection. In this case, however, animal studies have not provided unequivocal support for the hypothesis with different studies showing minor or no influence of inflammation on GID-like behaviors in rats (Lane et al., 2008; Soderstrom et al., 2008).

Recent studies have shown that LIDs might involve serotonergic terminals that innervate the striatum (Carta et al., 2010). Specifically, these striatal serotonergic terminals can take up L-dopa and convert it to dopamine. Once the dopamine is released by the serotonergic terminal, it cannot be inactivated as these neurons lack presynaptic dopamine transporters (Carlsson et al., 2007; Carta et al., 2007, 2010) which results in abnormal activation of dopamine receptors on striatal neurons. It is known that VM grafts can contain substantial numbers of 5-HT neurons, but concerning GIDs, this mechanism of genesis seems less likely as some of the patients exhibit GIDs despite not taking L-dopa (Lane et al., 2006). Furthermore, some transplant patients showed an improvement in LID together with worsening GID (Hagell et al., 2002; Hauser et al., 1999). In animal models, GID-like movements can occur in the absence of serotonergic neurons in the implants and when the endogenous serotonin system is lesioned, which further argues against an important role for serotonergic neurons in clinical GIDs (Lane et al., 2009).

### *Trial design*

The design of the trials to investigate the efficacy of fetal VM grafts does not simply concern whether open-label or double-blind imitation surgery trials are the best way to assess graft efficacy. An equally important issue is whether the latter trials were powered to show any significant transplant-mediated effects. In similar studies in PD using GDNF infusions to treat the underlying disease, sample sizes of 17 were felt by some to be inadequate to show significant effects. Thus some commentators have argued that trials with such small numbers of cases are never going to be free of Type II errors and as such the trials are flawed in their design (Barker, 2006; Hutchinson et al., 2007; Matcham et al., 2007).

Finally, it is important to remember that cell therapy approach, as discussed above, is only ever designed to replace the dopaminergic neurons that are lost within the nigrostriatal pathway, and PD is, of course, a disorder in which the pathology extends well beyond this system (Braak et al., 2004; Lang and Obeso, 2004). Whilst this is undoubtedly true, it is well known that dopaminergic drug therapies can radically improve the symptoms and signs of PD at all stages. Interestingly, the optimal control of the

dopaminergic responsive elements of PD can indeed even improve some of the non-dopaminergic features such as sleep disturbances, daytime somnolence, and fatigue (Honig et al., 2009). Thus using cells to replace the lost dopaminergic system is never going to cure patients of PD, but like dopaminergic drugs the cell therapy approach has the potential to dramatically improve their clinical state and quality of life (Honig et al., 2009).

## Neuropathological changes in grafts raise new concerns

As discussed above, the safety and efficacy of VM grafting for PD have become major points of discussion, but another important observation has further complicated the future of neural grafting in PD. This is the demonstration in post-mortem studies that neurons in fetal VM transplants contain alpha-synuclein pathology (Kordower et al., 2008a, b; Li et al., 2008b, 2010). This suggests that the grafts might ultimately undergo the fate of the patients' own dopaminergic neurons and succumb to the disease process of PD. The relevance of these recent post-mortem studies is not confined to the debate of VM grafting for patients with PD, but it throws up a whole series of questions related to what causes PD and how does the pathological spread of the disease occur (Brundin et al., 2008, 2010). However, that discussion lies beyond the scope of this review, and we will just briefly review the findings of post-mortem pathology in relation to the future of neural transplantation in PD.

In two initial back-to-back papers, Li et al. (2008b) and Kordower et al. (2008a) reported on Lewy bodies in nigral transplants in patients who had been grafted over a decade before they died. Li and coworkers described two patients who underwent the first of two sequential transplantation surgeries at age 48 ("patient 3" in the series) and 43 (patient 8), with 11- and 5-year disease history, respectively, of PD. Their second transplantation surgery took place 2–4 years later. They received dissociated fetal VM tissue from 6–8-week-old aborted embryos into either the putamen only or both caudate and putamen. Patients 3 and 8 received immunosuppression with prednisolone and cyclosporine for 24 and 18 months and azathioprine for 20 and 6 months, respectively. Patient 3 was the first in the Lund series reported to show significant improvement (Lindvall et al., 1990) whereas patient 8 showed minimal clinical benefit from the graft surgery (Hagell et al., 1999). At post-mortem, 13–16 years after their respective first transplantation surgeries, both patients exhibited numerous surviving and integrated grafted neurons. Patient 3 had an estimated 12,100–29,500 grafted tyrosine hydroxylase (TH, the dopamine-synthesizing enzyme)-positive neurons in each of the multiple injection tracks (Li et al., 2008b), indicating that he probably had over 100,000 surviving grafted TH neurons in total in each putamen. In both patients, the grafted neurons contained alpha-synuclein- and ubiquitin-positive Lewy bodies (Fig. 1) and Lewy neurites, morphologically

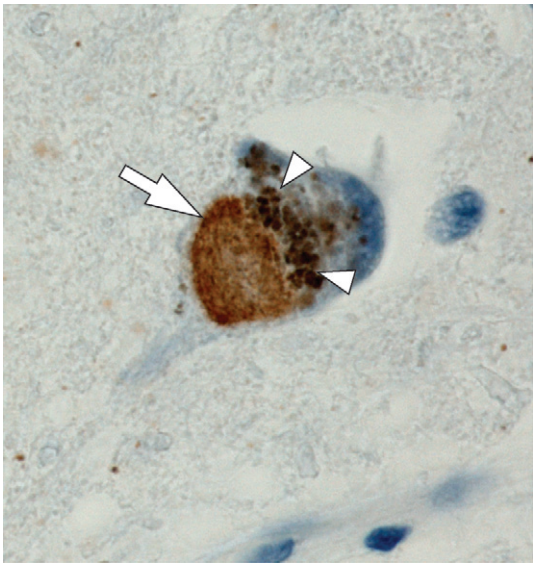

Fig. 1. Alpha-synuclein immunoreactive (arrow) Lewy body located in a neuron containing neuromelanin pigment (arrowheads) inside a VM graft in patient 3 from the Lund transplant series, as described in previously published papers (Li et al., 2008b, 2010). Photograph kindly provided by Dr J.-Y Li.

indistinguishable from those seen in surviving neurons in the host substantia nigra pars compacta. In young healthy individuals, cytoplasmic alpha-synuclein, even in non-aggregated form, is not detectable in the cell body. With normal aging it is upregulated and can be visualized by immunohistochemisty in an increasing proportion of nigral TH neurons (Chu and Kordower, 2007). In patient 3, the proportion of TH neurons with detectable amounts of cytoplasmic alpha-synuclein in the cell body was 40% in the graft done 12 years ago, and 80% in the graft done 16 years ago, suggesting gradual changes in alpha-synuclein levels. Finally in this paper, it was shown that the Lewy bodies in the grafts also stained with an antibody to alpha-synuclein phosphorylated at Ser129, indicating disease-related, post-translationally modified, and aggregated alpha-synuclein (Anderson et al., 2006). Whilst microglia accumulated around the graft in patient 3, they did not show significant activation (Li et al., 2008b).

Kordower et al. reported on a 61-year-old patient with a 22-year history of PD who had received bilateral solid fetal VM transplants to the putamen from four, 6.5–9-week old, embryos 14 years earlier (Kordower et al., 2008a). The patient improved clinically for a period of ~11 years after transplantation and then gradually deteriorated. Post-mortem analysis at death showed good graft survival with extensive TH innervation of the host striatum (Kordower et al., 1996). Kordower and coworkers also found cytoplasmic, aggregated and neuritic alpha-synuclein in grafted neurons. Moreover, the aggregates were ubiquitinated; some with the appearance of Lewy bodies were seen in grafted neurons. The transplants were also filled with activated microglia to a level far greater than that seen in the host striatum. The investigators did not find any Lewy bodies in grafted neurons in two brains from patients who had died only 4 years after surgery (Kordower et al., 2008a).

Simultaneously with the two reports describing Lewy bodies in grafted neurons, a third paper reported no neuropathological changes in transplants in three patients operated 9–14 years before dying (Mendez et al., 2008). Later, however, the same team of investigators has reported that they now have found some Lewy bodies in at least one of the 9-year-old grafts (Isacson, personal communication and Kordower et al., 2008b).

The initial reports on Lewy bodies in grafted neurons have been substantiated by more detailed descriptions and additional cases. Kordower and collaborators found Lewy bodies in one additional case 14 years after surgery (Kordower et al., 2008b). The Lewy bodies in grafted neurons have been found to be positive for thioflavin S, indicating that they contain the expected beta-pleated sheet structures (Kordower et al., 2008b; Li et al., 2010). Electron microscopic studies suggest that the Lewy bodies contain alpha-synuclein fibrils, further substantiating that they are indistinguishable from those seen in PD (Li et al., 2010). Interestingly, a detailed quantification of the proportion of pigmented (i.e., dopaminergic) neurons in the grafts that contained Lewy bodies in one of the patients, who received implants in two surgical sessions spaced by 4 years, revealed that they appear gradually. Thus, in the 12-year-old graft 1.9% of the dopaminergic neurons had Lewy bodies, whereas the corresponding number was as high as 5.0% in the 16-year-old cells (Li et al., 2010). Thus the proportion of neurons with Lewy bodies in 12–16-year-old transplants is similar to the 3.6% reported for the substantia nigra pars compacta in PD patients (Greffard et al., 2010).

The unexpected findings of Lewy bodies in implanted neurons in patients with PD suggest that the pathological process can affect young dopaminergic cells. Why this occurs is not known—it may relate to disease spread into the transplant via a prion-like mechanism (Brundin et al., 2008, 2010) or alternatively upregulation of alpha-synuclein in cells surviving in the inflamed environment of the grafted brain could eventually lead to aggregation of the protein (Brundin et al., 2008). Furthermore it remains unclear, though, how significant these findings are to the future use of cell therapies in PD. It is still only in a

small proportion of cells and with an uncertain significance to the viability and efficacy of the graft. However, in addition to findings of increased cytoplasmic levels of non-aggregated alpha-synuclein that might indicate early aging, other observations suggest that even the transplanted dopamine neurons that do not display Lewy bodies eventually undergo some form of degenerative changes. First, the expression of the dopamine transporter (DAT) is downregulated in the grafted neurons several years after surgery (Chu and Kordower, 2010; Kordower et al., 2008b), in contrast to what is seen in younger grafts. Previous *in vivo* brain scan studies suggest that reductions in DAT in the striatum may be a pathological sign that precedes the development of PD (Sommer et al., 2004). Second, Kordower and coworkers reported that some of the neuromelanin-bearing neurons in the graft no longer express TH in the long-term surviving implants, which might also be a sign of neuronal dysfunction (Chu and Kordower, 2010). The same grafted neurons expressed apparently normal levels of vesicular monoamine transporter 2 (VMAT2) suggesting that there was not a general downregulation of protein expression, but that DAT and TH were particularly affected (Chu and Kordower, 2010). Taken together, the increase in cytoplasmic alpha-synuclein levels, as well as the decreased levels of DAT and TH, suggest that a significant proportion of the grafted dopamine neurons undergo some kind of degenerative change when they have been present in the PD brain environment for over a decade.

## Cell transplantation in PD—where do we go from here?

Evidently, cell-based therapies for PD using fetal VM have had a chequered history—showing in some cases significant long-lasting benefits, whilst in other patients troublesome side effects have arisen. As described above, there is now accumulating data to suggest that some of the critical factors in the success (or failure) of these fetal VM transplants for patients with PD have been identified and that the field is ready to move forward again (although not all would agree—see Olanow et al., 2009). This will initially be with a further round of fetal VM transplantation trials before the next generation of stem cell-based treatments for PD are considered. Before performing the next series of fetal VM transplant studies, there is a need to address several practical issues related to tissue procurement, dissection, and storage, which we describe in the following sections and this programme of work, including the new clinical trials, has now been funded by the European Commission (TRANSEURO).

### *Ethics and tissue procurement of fetal neural tissue for grafting*

The use of fetal brain tissue for clinical transplantation purposes is coupled to two major issues. First, whereas some countries have adopted the view that donor tissues obtained from fetuses should be treated the same way as tissues or organs from dead adults (i.e., governed by transplantation law and including informed consent from the woman undergoing abortion), others prohibit the use of fetal tissue for transplantation on ethical or religious grounds (Boer, 1994). Second, even when the use of fetal tissue is accepted by society, practical issues make the coordination of clinical trials difficult. The majority of clinical trials have used VM tissue from 2–6 embryos per surgical session. In most major hospitals, induced abortions are common practice, but only dopaminergic neurons from a minority of embryos/fetuses are suitable for transplantation. The age of the donor embryo is crucial. Thus, VM tissue from embryos smaller than 14 mm crown–rump length has proven difficult to dissect with contamination of non-neural tissue. On the other hand, if the tissue is taken from an embryo/fetus larger than around 28 mm crown–rump length, the survival of dopaminergic neurons is poor when the cell

suspension transplantation technique is used (Freeman and Brundin, 2006). In accordance with ethical guidelines, when and where the abortion is performed must not be affected by the desire to use fetal tissue for transplantation (Boer, 1994), which contributes to the fact that several potential donors fall outside the optimal developmental time window. Moreover, in a significant proportion of cases the embryo/fetus is destroyed during the abortion, making it impossible to identify and dissect the VM. Taken together, the above factors make it difficult to predict if sufficient amounts of donor tissue will be available for transplantation to take place. Most clinical grafting programs that were active up until the publication of the NIH-sponsored trials struggled with this problem, and on several occasions planned surgeries have had to be postponed at the very last minute.

During recent years the practicalities of using fetal tissue derived from induced abortions has been complicated further by yet one more factor. The clinical trials performed so far have utilized VM tissue obtained from routine induced surgical abortions performed using vacuum aspiration, quite often under ultrasound guidance (Gustavii, 1989). During the past decade, the use of medical abortions has increased dramatically, with a concomitant decline in the use of surgical abortions. Mifepristone was introduced in Europe in 1988 as an anti-glucocorticoid and anti-progesterone drug that can induce terminations of early pregnancies (Schaff, 2010). Today it is often used to induce abortions during the first 9 weeks of gestation, i.e., the time window suitable for harvesting immature dopamine neurons for transplantation. Depending on which country in Europe one looks at, in extreme cases mifepristone accounts for over 50% of all induced abortions (Schaff, 2010), and the proportion is higher during the first 9 weeks. As a result the number of surgical abortions of embryos/fetuses that are suitable as donors in clinical transplantation trials has decreased dramatically. Whereas the supply of potentially useful neural donor tissue from surgical abortions was previously not a major hurdle to clinical grafting, today it is very difficult or impossible to run a clinical program that relies only on surgical abortions. As a result it will be necessary to examine the use of VM donor tissue from medical abortions in the future. The potential drawback with this is that the viability of the brain donor tissue might be compromised during the medical abortion. The time taken from the start of the medical induction of the abortion to when the fetus is expelled is several hours (Schaff, 2010), and this might mean that the immature neurons have been anoxic for excessively long periods before they can be harvested and prepared for transplantation. Whether tissue from medical abortions can be used as donor material, when grafting dopamine neurons, is currently being explored. Research teams are transplanting the tissue to immunosuppressed rodents and comparing the average yield of dopamine neurons, and variability between donors, with that seen using tissue from surgical abortions. The outcome of these studies will be vital in determining whether transplantation using donor tissue from abortions has a future or not.

### *Tissue dissection*

The dissection of fetal midbrain tissue is typically based on morphological landmarks where the rostral limit is just caudal to the mesencephalic–diencephalic border, the caudal limit is at the tuberculum interpedunculare of Hochstetter. The lateral border is set in the basal plate between the floor plate and sulcus limitans, such that the dissected piece includes the ventral third of the neural tube (Freeman and Brundin, 2006). In recent years, many key regulators of dopamine neurogenesis, such as Lmx1a, Ngn2, FoxA2, and Pitx3 have been identified (Andersson et al., 2006, 2007; Kele et al., 2006; Maxwell et al., 2005) and spatiotemporal analysis of these transcription factors in the human fetal midbrain (Hebsgaard et al., 2009; Nelander et al., 2009) has aided in refining the dissection technique. A typical dissection of a human embryo at a stage equivalent to

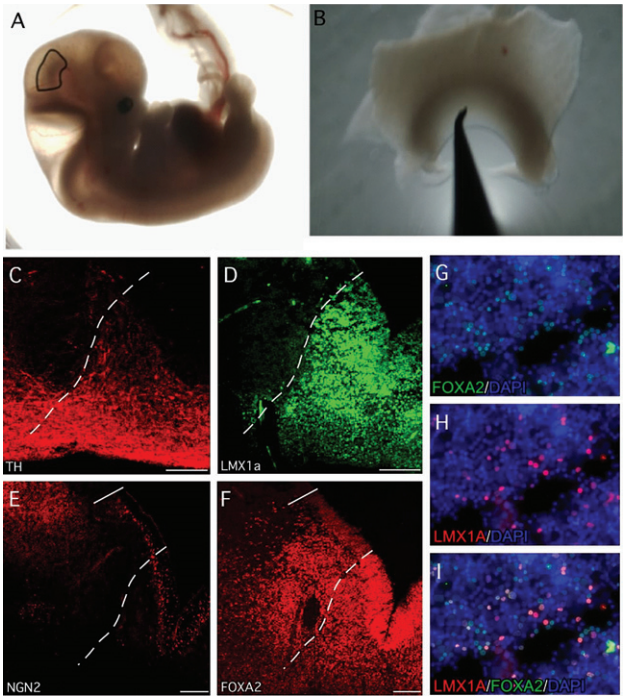


Fig. 2. Photograph illustrating a standard dissection. (a)VM from a human embryo that includes the entire mesencephalic dopamine domain. (b) A cross-section at the level of midway through the midbrain of similar stage embryo showing the dopamine domain as marked by TH (c), LMX1A (d), NGN2 (e), and FOXA2 (f). Cytospin of the resulting cell preparation showing a high proportion of DA progenitors as identified with the co-expression of LMX1a and FOXA2 (g–i). In the latter panels the cell nuclei are visualized using DAPI staining (blue). Photographs kindly provided by Jenny Nelander, Lund University. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this book.)

mid-dopaminergic neurogenesis is shown in Fig. 2a. This dissection is performed with the aim of including the maximum number of dopamine neurons and their progenitors (Fig. 2b) whilst excluding as many non-dopaminergic cells as possible. The resulting cell suspension from such dissections performed on CRL 18 embryos yields approximately 5% post-mitotic dopamine neurons as determined by the number of cells immunopositive for TH, 40% FoxA2-positive cells, and 25% dopamine progenitors identified by co-expression of Lmx1a and FoxA2 (Fig. 2c). Thus, it is clear that better dissections of the developing human midbrain dopaminergic neurons is now possible with resultant higher yields of the important population of substantia nigra dopamine neurons.

### *Storage of neural donor tissue for transplantation*

For all the reasons described above, it is likely that accessibility to suitable fetal donor tissue on a given planned day of surgery will be a limiting factor for future clinical transplantation programs. Even if the amount of suitable tissue available during one week is sufficient, coordinating the neurosurgical operation with tissue access will probably be very challenging. This means that development of effective tissue storage methods is now exceptionally important. This would allow pooling of brain tissue from abortions taking place on different days and thereby facilitate surgery. There are other advantages with tissue storage. For example, the delay period allows for more

extensive bacteriological and virological examination of the donor tissue. If immunological matching between donor and host proves to be important in the future, the storage period can also be used to have time for this. The time in storage can be used to expose the tissue to factors that promote its survival or, e.g., increase axonal growth. Finally, in some programs it has been deemed important that the woman undergoing abortion is not dependent upon the healthcare decision when she makes the decision to donate the tissue. Introducing prolonged tissue storage makes it possible for her to give her informed consent several days after the abortion. So far three different methods of storing embryonic VM tissue prior to grafting have been tested: explant or cell culture; freezing; and refrigeration.

Regarding the use of cell culture as a means of storage, the two main options are dissociated and solid tissue cultures. Dissociated monolayer cell cultures are of limited usefulness as a storage method for fetal dopamine neurons, because when the neurons have matured *in vitro* they are very prone to die upon re-dissociation from the culture well (Brundin et al., 1988). Tissue explant cultures, on the other hand, are more versatile in this context. If the dopamine neurons mature within a small solid tissue piece they can be harvested and grafted without their integrity being disturbed. Freed and coworkers used this method to store donor tissue for 1–4 weeks in one of NIH-sponsored clinical studies (Freed et al., 2001). One advantage is that the cells can be exposed to growth factors during the culture period (Clarkson et al., 2001), which might enhance their survival and function after grafting. A potential disadvantage, however, is that the culture period might significantly alter the relative proportions of different cell types in the VM graft tissue, thereby affecting the functional efficacy of the graft. Precisely this phenomenon has been suggested (Bjorklund et al., 2003) to contribute to the GIDs observed in the first NIH-sponsored trial (Freed et al., 2001), although there is still no experimental or clinical evidence that grafts of explant cultures of VM give rise to other functional effects than those of freshly prepared VM tissue.

Whereas freezing of fetal VM tissue down to –90–196°C allows for essentially unlimited storage periods, current protocols unfortunately result in significant additional loss of dopaminergic neurons when the tissue is grafted (Frodl et al., 1994). Despite this, freezing was used to store tissue in one of the early open-label clinical grafting trials in PD (Spencer et al., 1992) which resulted in very poor transplant survival as evidenced by post-mortem examination of one of the implanted patients (Redmond et al., 1990). Another more versatile method for tissue storage based on the same principle of slowing down the metabolism and maturation of the fetal neural cells is “hibernation”, which essentially involves refrigeration of the tissue to 4°C in defined media. This method has proven very easy to use and is highly successful when it comes to storage of VM tissue. When rat VM tissue is hibernated at 4°C for 2–3 days, the survival of dopamine neurons upon subsequent intracerebral grafting is equivalent to what is obtained with fresh tissue (Nikkhah et al., 1995; Sauer and Brundin, 1991). The method has already been used to store tissue used in clinical trials (Freeman et al., 1995; Mendez et al., 2000; Olanow et al., 2003). When the VM tissue is stored for 5–12 days, a gradual drop-off in the survival rate of the grafted dopamine neurons is seen (Nikkhah et al., 1995; Sauer and Brundin, 1991). The reduction in survival of immature dopamine neurons can be counteracted by the addition of lipid peroxidation inhibitors called lazaroids (Grasbon-Frodl et al., 1996) and the immunophilin ligand FK506 (Castilho et al., 2000). It has also been reported that the addition of glial cell line-derived neurotrophic factor (GDNF) to the hibernation medium results in improved graft survival (Apostolides et al., 1998), but this claim has been challenged (Brundin et al., 1999; Petersen et al., 2000). Possibly, GDNF, which is known to be protective to dopamine neurons (Zigmond, 2006), cannot exert beneficial effects at low temperatures as it is not effective on dopamine neurons that are metabolically quiescent (Brundin et al., 1999). Notwithstanding the unclear effects of

GDNF, it has been added to hibernation medium used in two series of clinical grafting trials (Mendez et al., 2000 and unpublished results). While hibernation of VM tissue is an effective storage method for a few days, and grafts obtained from such tissue clearly can exert functional effects in animal studies (Nikkhah et al., 1995; Sauer and Brundin, 1991), it remains to be studied whether the hibernation period alters the relative cell composition (including non-dopaminergic neurons or glial precursors) in the grafts. This will be important to know before future clinical grafting studies employing long-term tissue hibernation are undertaken.

### *Stem cell-derived neurons for grafting in PD*

If cell transplantation is to become a widely used therapy for PD, embryo/fetus-derived dopamine neurons are unlikely to constitute the main source of cells, even if all the practical problems discussed above are solved. There are simply too many variables concerning the tissue that cannot be finely controlled. Moreover, the protocols for procuring tissue are too labor-intense and are still not accepted for ethical reasons in many countries. One solution would be to develop methods that allows one to generate transplantable cells from renewable cell sources, such as stem cells that can generate large numbers of quality-controlled dopaminergic neurons (Fig. 3). This concept has been discussed widely for the past decade and has been the topic of numerous review articles. Indeed PD is often mentioned as one of the disorders where stem cell-derived cells might be applied clinically. There are several candidate stem cell types that can be subdivided into three main categories: lineage-specific stem cells, pluripotent stem

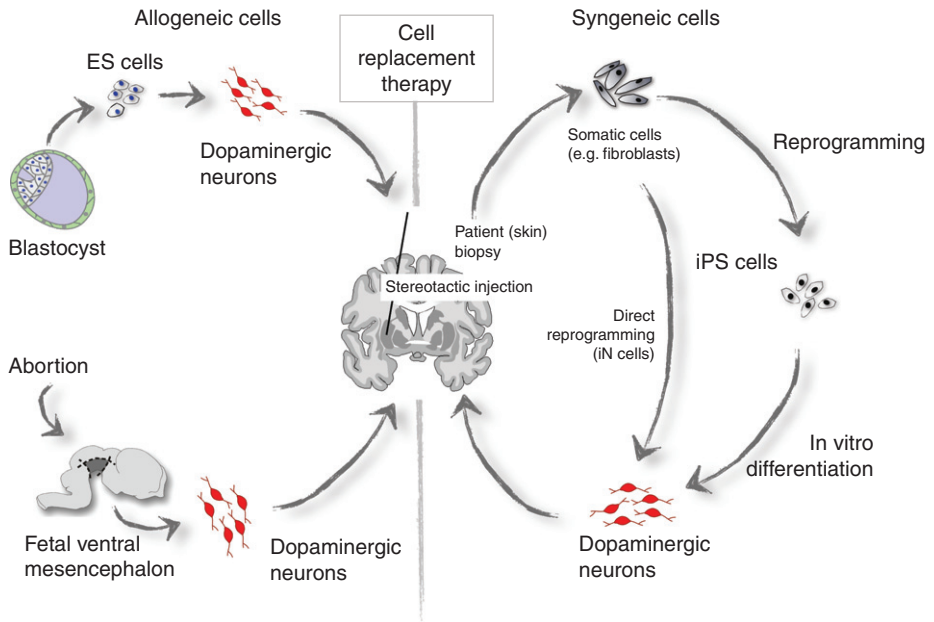


Fig. 3. This schematic drawing describes the main sources of potential donor tissue that can be used for transplantation in PD. The left part of the figure depicts hESCs and fetal tissue sources, which both result in allografts. The right part of the figure illustrates the recently discovered iPS and iN cells that could be sources of syngeneic "personalized" donor tissue originating from the patient. The schematic drawing is modified from an original illustration made by Dr Laura E. Allan et al. (2010), and the brain drawings were kindly provided by Bengt Mattsson, both at Lund University.

cells, and re-programmed somatic cells. In the following sections we describe the pros and cons of each stem cell type. During recent years, a set of criteria have been defined that stem cell-derived dopaminergic neurons need to fulfill before they can be considered for clinical application. These criteria are outlined in Table 1.

Table 1. Ideal characteristics of a dopaminergic cell transplant derived from a stem cell source for possible use in patients with Parkinson's disease

*Dopaminergic characteristics of cells in vitro*:

- Neuronal differentiation with morphology typical for midbrain dopaminergic neurons.
- Differentiation that is efficient such that sufficient numbers (e.g., 100,000 dopaminergic cells per grafted side of brain) of cells could be derived from a reasonable starting number of cells.
- The dopaminergic cells should express standard substantia nigra pars compacta markers such as TH; DAT; VMAT-2; and Girk-2.
- The dopaminergic cells so derived should have an appropriate expression profile in keeping with normal dopaminergic neuronal development including Pitx3, Nurr 1, En1/2, etc.
- The cells must show neurophysiological properties similar to that seen in mature nigral dopaminergic neurons and release dopamine.

*Other cells in the transplant:*

- The number of other neuronal cells derived from the stem cell source should be defined including Girk2-negative (non-nigral) dopaminergic neurons and serotonin neurons.
- The number of proliferating cells must be defined (e.g., using Ki-67) and the presence of such cells quantified and shown to be lost after short times to prevent graft overgrowth/tumor formation.
- The absence of cells of ES/iPS cell origin, using markers such as Nanog, Sox 4, must be demonstrated.
- The karyotype of the cells must be shown to be normal and stable.

*Cell behavior in experimental models of* PD:

- The dopaminergic cells so derived must continue to express the above nigral markers for long periods of times (i.e., months) after grafting into the adult brain.
- The cells should extend processes and innervate the striatum with evidence of synapse formation.
- The transplanted cells should have functional effects equivalent to that reported for dopaminergic cells derived from primary VM tissue (Grealish et al., 2010).
- The cells grafted must not form tumors or express neither: (i) markers of early stem cells (e.g., Nanog; Oct 4, etc.) nor (ii) markers of cell proliferation beyond the immediate post-transplant period.

Briefly one can summarize the challenges facing stem cell therapy in PD into three areas. The first challenge is to develop protocols that efficiently promote the stem cells to differentiate *in vitro* into dopamine neurons of the midbrain phenotype. Second, it will be necessary to devise techniques to ensure that the differentiated dopamine neurons survive intracerebral grafting and continue to function as dopamine neurons after the surgery. Experimental studies have shown that presence of a high proportion of dopamine neurons in cultures derived from stem cells or immortalized cell lines does not necessarily mean that the same cells will survive (Brederlau et al., 2006), or alternatively retain their dopaminergic phenotype (Paul et al., 2007), after grafting to the adult brain. Third, as we discuss further in later sections, it is essential that the stem cell-derived cells do not continue to proliferate excessively and generate tumors after transplantation to the brain (Li et al., 2008a).

### *Lineage-specific stem cells as a source of dopamine neurons*

Neural stem cells exist at all rostro-caudal levels of the developing neural tube and in discrete regions of the adult brain (Gage, 2000; Kintner, 2002). Both fetal and adult neural stem cells are self-renewing and give rise to the three major central nervous system (CNS) cell types: neurons, astrocytes, and oligodendrocytes *in vivo* as well as *in vitro* (Alvarez-Buylla et al., 2001; Kintner, 2002).

In the early 1990s, it was found that it is possible to expand neural stem and progenitor cells from the fetal and adult CNS *in vitro* for long periods without the use of immortalization factors, commonly as free-floating aggregates of cells, termed neurospheres (Reynolds and Weiss, 1992; Reynolds et al., 1992) or other growth factor-stimulated attached cultures (Buc-Caron, 1995; Laywell et al., 2000; Palmer et al., 1999). The neurosphere culture has long been the most commonly used expansion system and it offers some obvious advantages: neurospheres are simple to

establish and maintain and they survive, integrate, migrate, and differentiate in the host brain after transplantation (Caldwell et al., 2001; Fricker et al., 1999; Rosser et al., 2000). However, neurospheres are heterogeneous in their cell composition and tend to lose neuronal differentiation potential over time (Anderson et al., 2007; Parmar et al., 2002; Suslov et al., 2002). In 2005, Conti and colleagues introduced the neural stem (NS) cell culture system (Conti et al., 2005). Like the neurospheres, NS cells are grown in defined serum-free conditions in the presence of the mitogens epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), but in adherent monolayered cultures as opposed to in free-floating aggregates. The NS cell cultures are highly homogenous with respect to morphology and neural progenitor marker expression; they show radial glia-like characteristics and retain their tripotent differentiation potential, including the capacity for neuronal differentiation even after prolonged expansion (Conti et al., 2005; Sun et al., 2008).

Both NS and neurosphere cultures can readily be established from the developing VM of both rodents and humans (Caldwell and Svendsen, 1998; Caldwell et al., 2001; Hebsgaard et al., 2009; Ostenfeld et al., 2002). Whereas dopamine neurons can be formed *in vitro* from short-term expanded cells from the VM (Parish et al., 2008; Studer et al., 2000; Yan et al., 2001) or immature cortex (Lee et al., 2010), multi-passaged cells show a limited ability to generate dopamine neurons, even after genetic manipulation to induce a dopamine neuron fate (Andersson et al., 2007; Caldwell and Svendsen, 1998; Chung et al., 2006; Roybon et al., 2008). The relative difficulty with which dopamine neurons can be differentiated from tissue neural stem cells can be attributed to the fact that expansion in EGF and bFGF tend to bias the cells to a GABA-ergic fate. An alternative explanation may be the special ontogeny of the midbrain dopamine neurons: a growing body of evidence shows that the dopamine neurons are uniquely derived from floor plate cells that become neurogenic only in the midbrain region (Bonilla et al., 2008; Hebsgaard et al., 2009; Joksimovic et al., 2009; Ono et al., 2007). Floor plate cells may require different conditions for expansion and thus they are not maintained using standard culture conditions with EGF and bFGF, resulting in a diminished capacity to generate dopamine neurons. Taken together, therefore lineage-specific stem cells are currently not the most versatile or promising source for generation of dopamine neurons.

### *Embryonic stem cells as a source of dopamine neurons*

Many of the limitations associated with expanded fetal and adult neural stem cells are overcome if one turns to an earlier stem cell type, the embryonic stem cell (ESC). The ESCs are self-renewing and pluripotent cells that are derived from the inner cell mass of the pre-implantation blastocyst (Fig. 3). Like the cells of the epiblast, ESCs can give rise to all cell types of the embryo and adult organism (Evans and Kaufman, 1981; Martin, 1981; Nichols and Smith, 2009). In the mouse, ESCs have the capacity to re-aggregate with the epiblast and contribute to the formation of new embryos including germline cells (Bradley et al., 1984).

In 1998, the first successful derivation of human ESCs (hESCs) was reported (Thomson et al., 1998) and subsequently many hESC lines have been derived. Human ESCs maintain the developmental potential to contribute to cells of all three germ layers, even after clonal derivation (Amit et al., 2000), but whether hESCs correspond to pre-implantation epiblast cells of the mouse or the slightly more differentiated post-implantation epiblast cells is debated (Nichols and Smith, 2009; Tesar et al., 2007). Nevertheless, it is clear that this cell type is of considerable interest for cell replacement therapies due to a virtually unlimited self-renewal capacity and pluripotent differentiation spectrum.

Over recent years, several protocols have been published describing the derivation of neural tissue from pluripotent human cells. Whereas the initial protocols utilized the ability of pluripotent cells to

spontaneously give rise to neuroectoderm (Reubinoff et al., 2001), several factors have been identified which promote lineage-specific differentiation including co-culture with stromal cells (Brederlau et al., 2006; Zeng et al., 2004), low-density culturing, and addition of fibroblast growth factors (FGFs) or inhibition of SMAD signaling (Chambers et al., 2009). Generation of several specific subtypes of neurons including midbrain dopamine neurons from hESC have been reported (Fig. 4) (Cai et al., 2009; Correia et al., 2007; Hong et al., 2008; Iacovitti et al., 2007; Ko et al., 2007; Park and Lee, 2007; Park et al., 2005; Perrier et al., 2004), and more efficient protocols are consistently being developed. From rodent studies, we know that correct mesencephalic identity of the transplanted dopaminergic neurons is important for proper survival, integration, reinnervation, and function of the cells after transplantation (Grealish et al., 2010). Since ESCs differentiate into neurons

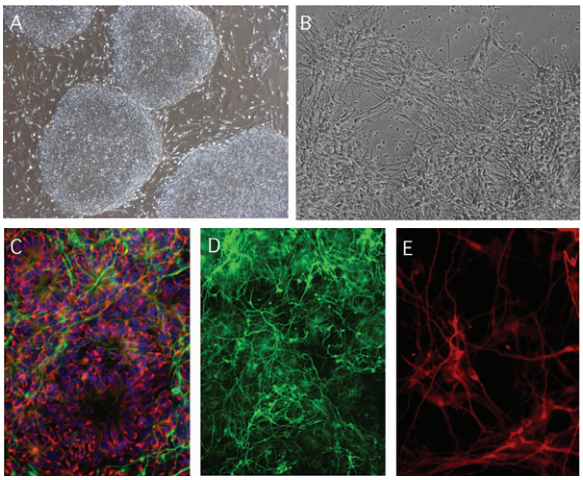


Fig.4. Undifferentiated human ES cells grow in colonies. (a) Upon plating the cells under neural conversion conditions. (b) They differentiate via a characteristic rosette-like stage corresponding to neuroepithelial cells *in vivo*. (c) The cells in the rosettes are nestin positive (c, red). Neurons are starting to be formed at the rosette stage (c, green) and the neuronal differentiation proceeds. (d) After 28 days of differentiation, TH neurons can be detected (e). Photographs kindly provided by Dr Agnete Kirkeby. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this book.)

with characteristics of all levels of the CNS, it is therefore important to establish that the hESC-derived dopamine neurons exhibit a correct VM identity to ensure functionality (Table 1). In ESC cultures, where positional identity is lost, VM character must be demonstrated with the combined expression of several midbrain markers such as Lmx1a/b, FoxA2, Nurr1, and Pitx3 in combination with TH (Fig. 4).

The unlimited differentiation capacity and the pluripotent differentiation capacity of ESCs make them promising candidates as a potential source of transplantable neurons. On the other hand, these characteristics are also what make ESCs difficult to work with. Consequently, there are several crucial issues to be resolved when it comes to hESC differentiation into functional dopamine neurons. For example, several studies have failed to demonstrate robustly that grafted hESC-derived neurons ameliorate behavioral deficits to the same degree as fetal VM-derived neurons (Christophersen and Brundin, 2007; Li et al., 2008). Part of this problem may be related to poor survival or loss of the dopaminergic neuron phenotype when the hESC-derived neurons are grafted into the adult brain, despite the same cells strongly expressing TH in culture and other hESC-derived cells surviving the surgery (Li et al., 2008a) (Fig. 5). The immunogenicity of hESC-derived cells may also prove to be an issue in their clinical translation (Li et al., 2008a), but it is unlikely to represent more of a problem than that encountered to date with primary fetal neural transplants. Most importantly, the capacity of hESCs to form a multitude of somatic cell types means that one needs to take special care to ensure the purity of the differentiated cultures that are to be used for grafting. Particularly non-neural cells and neural cells of incorrect phenotype should be avoided, either by more efficient differentiation protocols or by positive or negative selection based on, for example, cell surface markers and fluorescence-activated cell sorting (FACS) (Hedlund et al., 2007, 2008; Nicholas et al., 2007; Placantonakis et al., 2009; Pruszak et al., 2009). Thus, a major obstacle to using ESC in a clinical

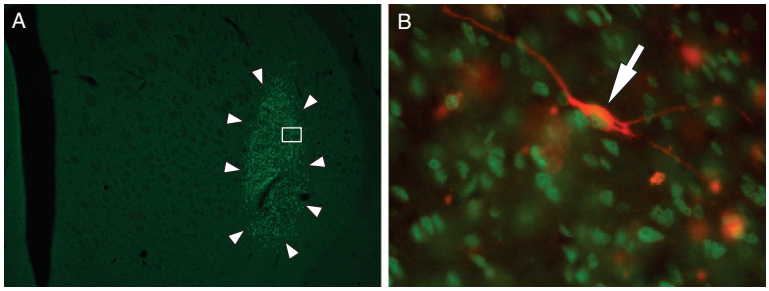


Fig. 5. Survival of human ESC-derived dopamine neurons after grafting to the rat striatum. (a) Low-power image showing a section through one hemisphere of a rat brain immunostained for human nuclear antigen. This immunosuppressed rat received an instrastriatal graft of hESC that had been differentiated into around 20% dopamine neurons. In the striatum, a hESC-derived graft, with immunopositive (green nuclei) cells, is clearly visible. The white box marks the region where the cells in Panel B are located. (b) High magnification of a dual immunostained image from the same graft as in Panel A. The green fluorescent cells are immunopositive for human nuclear antigen, whereas the red cells are TH-positive, and most likely dopaminergic. Photographs kindly provided by Dr Asuka Morizane, when at Lund University. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this book.)

setting is their capacity to form teratomas and neural overgrowth in the host brain. During the coming years, we need to gain a better understanding of how to control and synchronize neural differentiation of hESCs and how to prevent proliferation of the cells after transplantation.

### *iPS cells represent a novel and interesting source of cells*

Several recent studies show that somatic cells can be re-programmed into pluripotent cells (reviewed in Nishikawa et al., 2008) or directly into mature cell types (Vierbuchen et al., 2010; Zhou et al., 2008). These exciting and groundbreaking findings open up new possibilities for cell replacement therapy, as it is now possible to generate patient-specific stem cells on demand.

In 2006, Takahashi and Yamanaka showed in a pioneering study that fibroblasts from adult mice can be re-programmed into pluripotent cells by expressing only four factors (Oct4, Sox2, Klf4 and c-Myc). The resulting cells, termed induced pluripotent stem (iPS) cells, are phenotypically and morphologically very similar to ESCs, are germline-competent and contribute to chimeras at a reasonable frequency (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). Human cells were subsequently also found to be possible to re-program into iPS cells using the same four factors (Takahashi et al., 2007) or Nanog, Lin28, Oct4, and Sox2 (Yu et al., 2007) suggesting that patient-specific, genetically compatible cells for transplantation can be derived from skin biopsies from PD patients (Soldner et al., 2009). The initial iPS cells were derived using techniques not compatible with clinical applications as the reprogramming depended on oncogenes. The reprogramming genes were delivered using viral vectors and the genes were inserted in multiple sites of the genome. Several developments in iPS cell derivation technology have already been made. The number of genes required for reprogramming has been reduced, and iPS cells have now been generated using non-integrating viruses (Stadtfeld et al., 2008; Yu et al., 2007), plasmid systems that result in single integration site and allow for subsequent excision of reprogramming factors (Kaji et al., 2009; Okita et al. 2010; Woltjen et al., 2009), and also by direct delivery of recombined reprogramming proteins (Lyssiotis et al., 2009). All these improvements makes the iPS cells more clinically relevant, but further technological advances are needed in order to produce safe cells under good manufacturing practice (GMP) conditions that can be used in a clinical setting.

As was hoped for, iPS cells have been shown to respond to the same developmental patterning cues as ESCs. They use the same transcriptional network to generate neuroepithelium and functionally appropriate neuronal types including dopamine neurons, albeit with reduced efficiency and increased variability (Cai et al., 2009; Chambers et al., 2009; Hu et al., 2010; Wernig et al., 2008). The iPS cells also have the same proliferative capacity as ESC. Therefore, they share the risks for incomplete and unsynchronized differentiation, coupled with risk of tumor formation, and neural overgrowth after grafting with ESC.

An alternative approach may be direct reprogramming from one somatic cell type to another. Proof-of-concept of this comes from a study performed by Melton and colleagues who re-expressed three key developmental regulators that reprogram differentiated pancreatic exocrine cells in adult mice into cells that closely resemble endocrine beta-cells (Zhou et al., 2008). Similarly, combinatorial expression of three neural-specific transcription factors was recently found to directly convert fibroblasts into functional neurons *in vitro*, and this new class of cells has been named induced neuronal (iN) cells (Vierbuchen et al., 2010) (Fig. 3). Although this is an exceptionally interesting finding, the field is in a very early era. For example, the iN cells generated so far have not been demonstrated to include dopamine neurons. This initial study clearly provides evidence for the principle of cellular reprogramming of terminally differentiated cells without reversion to a pluripotent cell stage, which is a very attractive idea for generating patient-specific transplantable cells that differentiate into functional neurons after grafting. Importantly, the method can circumvent the issues with uncontrolled proliferation and incomplete differentiation. However, the method introduces one new problem: since the reprogrammed cells do not proliferate, very large numbers of reprogrammed neurons must be generated for each patient in order to provide sufficient material for transplantation. Furthermore, it is not inconceivable that PD patient-derived (thereby possible expressing PD susceptibility genes) iN cells or iPS cell-derived neurons might degenerate after grafting due to PD, in analogy to the patient's own dopamine neurons

## Safety and regulatory issues for clinical application of stem cells in the brain

Transplantation of neurons derived from a highly proliferative population of stem cells into the brain involves potential safety risks, which we have reviewed extensively elsewhere (Li et al., 2008a), mainly associated with the possible inclusion of proliferating cells that can form tumors. In any stem cell-based transplantation therapy, regardless of the target organ or tissue, tumor growth is a major safety concern. For stem cell therapy in the brain, it is a particularly grave concern. The confined space of the intracranial cavity coupled to the vital functions coupled to many brain structures means that even modest or slow tumor growth in this locale can have neurologically disastrous and potentially lethal consequences. The issue of graft-derived neural tumors was recently in the spotlight after the publication of a report of multifocal brain tumors in a boy suffering from ataxia teleangectasia who was treated with intrathecal and intracerebellar injections of neural stem cells (Amariglio et al., 2009). Furthermore, over the past few years the Californian biotech company Geron Corporation has worked hard to convince the Food and Drug Administration about the safety of their hESC-derived oligodendrocytes (GRNOPC1) and their suitability for use in a transplantation therapy for spinal cord injury. In particular, fears of tumor growth and cysts forming in the grafts have been the source of great scrutiny (DeFrancesco, 2009).

One possible cause for continued proliferation of stem cell-derived grafts, even after dedicated efforts to differentiate the cells into neurons, is the appearance of chromosomal aberrations in the stem cell cultures. While chromosomal

aberrations occur commonly in rapidly dividing cells, most of them result in cell death. Only rarely are the cells viable, and in a few of these cases the aberration provides the cells with an advantage (e.g., upregulation of a growth factor receptor) that makes them proliferate more rapidly. It is well known that hESC cultures can accumulate chromosomal changes, particularly in chromosomes 12 and 17, and that the abnormal cells often outgrow their normal neighbors (Buzzard et al., 2004). A recent study showed that among different neural stem cell lines derived from human fetal brain, 24% developed trisomy in chromosome 7 and 5% exhibited trisomy in chromosome 19 (Sareen et al., 2009). While these particular chromosomal changes did not result in tumor growth when the cells were grafted to the brain, they highlight the need to regularly monitor the cytogenetics of all cell lines considered for clinical use. Even epigenetic changes, e.g., alterations in gene promoter methylation (Maitra et al., 2005), in stem cell cultures can lead to quantitative changes in gene expression and growth selection of a subpopulation of cells that is more prone to tumor formation.

However, it is not sufficient to avoid changes at the chromosomal level in order to avoid tumor growth in stem cell neural grafts. Clearly, cells with normal chromosomes and epigenetic patterns also have the potential to proliferate. Thus, any remaining pluripotent stem cells in "differentiated" hESC cultures must be avoided as they can continue to divide and form teratomas upon transplantation (Brederlau et al., 2006). When grafting hESC-derived dopaminergic neurons, even residual neuroepithelial progenitors derived from stem cells, and present in the graft tissue, can continue to divide after intracerebral transplantation and give rise to large, and potentially harmful, tissue masses (Sareen et al., 2009). There exist several strategies that can be used to enrich the differentiated cell population in stem cell cultures or to deplete undifferentiated/proliferating cells from the same cultures. Briefly, they include the use of pharmacological agents that kill proliferating cells: cell sorting using FACS or magnetic activated cell sorting (MACS), and genetic manipulation of intracellular signaling pathways that govern cell proliferation. We recently reviewed these options in greater length elsewhere (Li et al., 2008a). The main challenge facing all these methods is that they must be 100% efficient. It is unacceptable to leave even one single pluripotent stem cell among the cells that are implanted into a brain. Whereas FACS and MACS are excellent techniques to sort cells used in laboratory studies, they typically suffer from the shortcomings that the cell surface markers are not fully exclusive/inclusive of the undesired/desired cell populations and that the apparatus exhibits slight experimental errors. Furthermore, FACS is a relatively traumatic procedure for neurons and as a result they can die during the procedure if they have long processes. Nonetheless, a recent study showed that using FACS to select cells based on their expression levels of three cell surface cluster of differentiation molecules, it was possible to selectively enrich hESC-derived neuronal cells and drastically reduce risk of tumor formation after grafting (Pruszak et al., 2009).

While all the safety issues we have just described are major scientific challenges, for them to be really relevant in a clinical setting they need to be addressed in the context of GMP (Unger et al., 2008). This adds yet another level of complexity because GMP requires that the issues are resolved using standardized protocols employing, e.g., animal-free (xeno-free) feeder cells and culture media in a setting with minimal risk of microbial infection. Although GMP-compatible protocols for hESC-derived dopamine neurons are currently being developed (Swistowski et al., 2009), the application of GMP rules and generation of clinical-grade hESC-derived dopamine neurons is costly, time consuming, complex, and constitutes a significant practical hurdle to the clinical application of stem cell therapy in PD. For example, not just the differentiation protocols, subsequent cell sorting and storage need to be performed according to GMP, but the hESC line itself (used to generate the dopamine neurons) has to be derived under GMP conditions in a certified laboratory.

## Future perspectives and concluding remarks

Cell transplantation still remains a very promising therapeutic approach for PD. While the initial promise and excitement of the early open-label trials with fetal VM grafts in PD has been diminished and undermined by the subsequent placebo-controlled, double-blind trials, we believe that the past 5–10 years has seen several developments that now make it possible once again to perform successful trials using fetal VM tissue in select groups of PD patients. By refining both the transplantation techniques and patient selection, we think that this approach will give more reproducible and marked symptomatic relief in the patients, and that the prevalence and severity of the unwanted GIDs will be reduced. Notwithstanding our optimism regarding fetal tissue transplantation in PD, we still consider it necessary to develop new alternative sources of donor tissue in the future, since fetal tissue can never be the basis of a widely accessible cell therapy for PD. The past decade has seen dramatic developments in stem cell research, including substantially increased knowledge in hESC biology and the developmental processes underlying midbrain dopaminergic neurogenesis, as well as the discovery of the reprogramming of somatic cells into stem cells and neurons that opens up the door to patient-specific "personalized" cells (Fig. 3). Taken together, this progress is likely to lead to the creation of stem cell-based therapies for PD. Without dampening our enthusiasm for these future stem cell therapies, we recognize that several safety issues and regulatory hurdles need to be negotiated during the coming 5–10 years before the PD patients will actually reap the benefits of this concerted research effort.

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## Abbreviations

| | |
|---|---|
| ADL | Activities of Daily Living |
| bFGF | basic fibroblast growth factor |
| DAT | dopamine transporter |
| ESC | embryonic stem cell |
| EGF | epidermal growth factor |
| FACS | fluorescence activated cell sorting |
| F-dopa | fluorodopa |
| GID | graft-induced dyskinesias |
| GMP | good manufacturing practice |
| hESC | human embryonic stem cell |
| iPS | induced pluripotent |
| MHC | major histocompatibility complex |
| NIH | National Institutes of Health |
| NS | neural stem |
| TH | tyrosine hydroxylase |
| UPDRS | Unified Parkinson's Disease Rating Scale |
| VM | ventral mesencephalon |
| PD | Parkinson's disease |
| PET | positron emission tomography |

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