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Pluripotent stem cells as new drugs? The example of Parkinson's disease

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ABSTRACT

Cell replacement therapy is a widely discussed novel concept of medical treatment. The increased knowledge in the stem cell field, particularly pluripotent stem cells, potentially provides powerful tools for this therapeutic concept. A large number of disease characterized by the loss of functional cells are potential candidates for cell replacement therapy and, in this regards, Parkinson's disease is of particular interest. It is one of the most prevalent neurodegenerative diseases caused by the loss of dopaminergic neurons in the *Substantia nigra pars compacta*. Pharmacological therapies are valuable but suffer from the progressive decline of efficacy as the disease progresses. Cell therapy application has emerged about two decades ago as a valid therapeutic alternative and recent advances in stem cell research suggest that pluripotent stem cell transplantation may be a promising approach to replace degenerated neurons in Parkinson's disease. Various sources of pluripotent stem cells (PSC) currently tested in animal models of Parkinson's disease have proven their efficacy in relieving symptoms and restoring damaged brain function. This review summarizes and discusses the important challenges that actually must be solved before the first studies of PSC transplantation can be undertaken into humans.

1. Introduction

Pluripotent stem cells (PSC) can be derived from embryos or through reprogramming of somatic cells (Tweedell, 2008). PSC can differentiate into virtually any cell type. This property of PSC is of interest as it enables: (i) studies on basic mechanisms of human cell differentiation, (ii) the use of PSC-derived human cells and tissues for *in vitro* functional studies, and (iii) the use of PSC-derived cells for therapy of diseases which are characterized by cell loss. The use of PSC for therapeutic purposes is particularly relevant for cell types which cannot be easily obtained otherwise. In this case, cells which are lost during the disease process are replaced by exogenously provided cells. Thus, cell replacement therapy represents a specialized form of transplantation.

Parkinson's disease (PD) is an interesting paradigm for cell replacement therapy. In PD, most motor features result from the selective loss of the pigmented neurons of the *Substantia nigra pars compacta* which provide a dopaminergic innervation to the medium spiny neurons of the *Striatum*. Thus, there is a good theoretical basis for the concept that implantation of dopamine-producing neurons at the appropriate anatomical location should provide a therapy for the disease (Hall et al., 2007). There is now abundant evidence in rodent models of PD that implantation of PSC-derived dopaminergic neurons leads to motor improvements. There is also one study demonstrating that such a strategy works in a primate model. Although PSC-derived neurons have not yet been used for human PD therapy, PD patients have already been implanted with human fetal mesencephalic progenitors (Lindvall and Bjorklund, 2004).

2. Pluripotent stem cells

At this point, embryonic stem cells (ESC) are the most reliable source for PSC, and differentiation protocols to obtain dopaminergic

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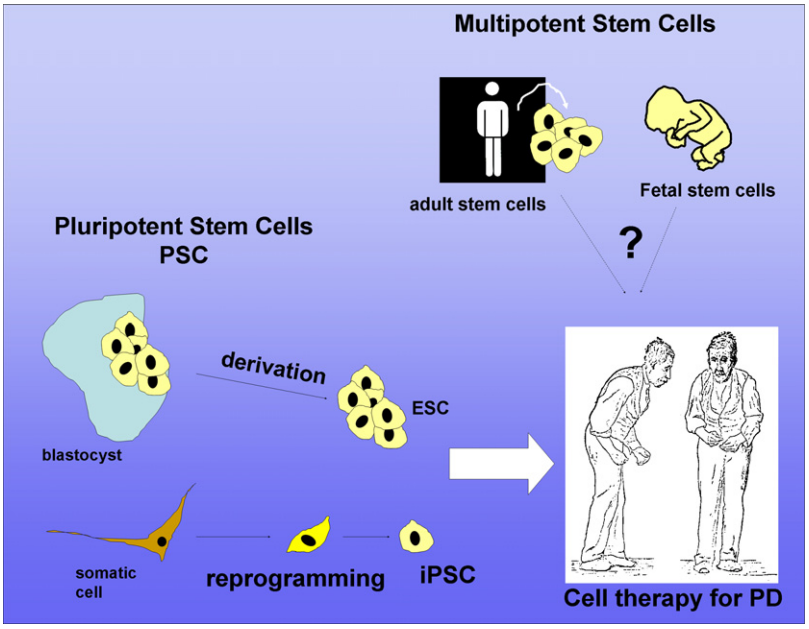

Fig. 1. Various sources for stem cells for PD's cell therapy. Pluripotent Stem Cells can be generated either from embryonic material (Embryonic Stem Cells) or from somatic cells reprogrammation (induced Pluripotent Stem Cells). Another source of stem cells (adult or fetal neural progenitors) are considered to be non-pluripotent but also emerged for their putative capacity to be differentiated towards dopaminergic neurons. (Parkinson patient, drawing by Sir W.R. Gowers, 1886.)

neurons have been mostly developed using ESC (Barberi et al., 2003; Perrier et al., 2004; Roy et al., 2006; Schulz et al., 2004; Yan et al., 2005). In general, stem cells from fetuses and adults do not fit the definition of pluripotency (Fig. 1). However, new reprogramming technologies induce pluripotent stem cells (iPSC) from fibroblasts and other adult cells (Wernig et al., 2008; Yamanaka, 2008). At this point though, the technology still: (i) does not produce cells fully identical to pluripotent ESC, and (ii) relies on transgenic expression of pluripotency factors in genetically modified cells. Nevertheless, the technology is promising and it is possible that on the long run, iPSC will replace ESC as the relevant source of PSC (Nishikawa et al., 2008; Wernig et al., 2008; Yamanaka, 2008).

3. Differentiation of pluripotent stem cells into target cells: the example of dopaminergic neurons

For various reasons, fetal mesencephalic progenitors transplantation has proved unsuccessful in most PD patients (see below). Indeed, whereas graft survival and function has been repeatedly demonstrated using ^{18}F-fluorodopa PET imaging, clinical benefit on parkinsonism has proved minimal and inconsistent from one patient to another. In addition, a significant proportion of recipients have exhibited disabling off-levodopa dyskinesia (Freed et al., 2001; Olanow et al., 2003). Mechanisms underlying this unexpected complication are unclear but may involve a non-physiological, excessive and heterogeneous dopaminergic stimulation provided by the striatum-implanted graft (Winkler et al., 2005). Thus, alternative cell sources need to be found. ESC are promising candidates for cell replacement therapy in PD as they can be maintained in laboratory culture and have the potential of *in vitro* differentiation into dopaminergic precursors and neurons. Initial protocols of ESC maintenance and cell differentiation were far from clinical applicability. The procedures were complicated, required the use of non-clinical grade and animal-derived products and ESC needed to be grown on murine feeder cells. Recently, however, this domain has much improved with procedures becoming simpler and eliminating, at least in part, animal products and co-culturing with animal feeder cells (Deierborg et al., 2008; Morizane et al., 2008). The yield of differentiated dopaminergic neurons is supposed to be acceptable (in the range of 40% in best studies), but the purity of the preparations as well as the culture and differentiation conditions still need further improvements in order to comply with requirements of good manufacturing practice and, thus, clinical applicability.

4. Regenerative medicine and Parkinson's disease

4.1. Cell therapy in animal models

Initial animal studies showed that transplantation of mouse ECS-derived dopaminergic progenitors into a rodent model of PD can produce improvement of motor symptoms.

A first study on grafted mouse ESC-derived dopaminergic neurons reported that around 22% of the TH-positive cells present in the original cell cultures survived after grafting (Kawasaki et al., 2000). A following study analyzed the functional improvement following transplantation of mouse ESC-derived neurons in rats with unilateral 6-OHDA lesions. It showed that the rats recovered completely symptoms after receiving grafts of Nurr-1 overexpressing cells (Kim et al., 2002). Many other animal experiments have analyzed behavioural recovery in rodents following transplantation of mouse ESC-derived cells. It is reviewed in (Hall et al., 2007). Only two studies reported a high level of functional recovery after grafting in rats with unilateral 6-OHDA lesions (Barberi et al., 2003; Rodriguez-Gomez et al., 2007). Rodriguez-Gomez et al. showed that transplanted mouse ESC-derived dopaminergic cells can survive long-term. A complete reversal of symptoms was observed at 4 weeks and remained stable up to 32 weeks following transplantation. Some dopaminergic neurons survived (≈5000 per host) and restored to 38% of normal the striatal levels of the dopamine transporter, which is a presynaptic marker of dopamine neurons. Transplantation studies have been also performed with dopamine neurons derived from human ESC. Generally the outcome has been less encouraging than with dopaminergic neurons derived from mouse ESC. The first transplantation

Table 1
Summary of the most important animal studies for ESC-based PD cell therapy.

Year	Study	ESC	Recipient	Dopaminergic neurons survival	Behavioral effects
2000	Kawasaki et al.	Mouse	Mouse	22%	Nd
2002	Kim et al.	Mouse ESC expressing Nurr-1	Rats (6OHDA)	Significant	Yes
2003	Barberi et al.	Mouse ESC	Rats (6OHDA)	Observed	Yes
2004	Ben-Hur et al.	Human ESC	Rats (6OHDA)	Observed	Modest
2005	Takagi et al.	Monkey ESC	Monkey (MPTP)	Observed	Yes
2006	Roy et al.	Human ESC	Rats (6OHDA)	High	High
2007	Rodriguez-Gomez et al.	Mouse ESC	Rats (6OHDA)	Observed	Yes

study of human ESC-derived neurons into rats with unilateral 6-OHDA lesions reported a modest reduction of symptoms (around 35–40%) (Ben-Hur et al., 2004). Another study reported an improvement in symptoms of up to 95% in rats with 6-OHDA lesions. The onset of the effect was unusually rapid and the extent of discovery exceeded that normally seen with e.g. grafted foetal midbrain neurons. Approximately 27,000 TH-positive cells were detected within the grafts following 10 weeks transplantation. A concern was the presence of dividing neural progenitor cells within the graft (Roy et al., 2006). The interpretation of the results from this study was however debated (Christophersen and Brundin, 2007). Finally, behavioural studies and functional imaging revealed that monkey ESC-derived dopaminergic progenitors acted as dopaminergic neurons after transplantation and improve neurological symptoms in a monkey model of PD (Takagi et al., 2005). Table 1 summarizes the main animal studies performed with ESC. In conclusion, further studies on transplantation of human ESC-derived neurons into parkinsonian models are necessary in order to fully evaluate the functional capacity of such cells.

4.2. Cell therapy in humans

Given the well-defined loss of a specific type of neurons (dopaminergic neurons) in a well defined region of the brain (*S. nigra*, Fig. 1), the concept of cell replacement therapy in PD has emerged already in the early 1980s. Importantly, this approach has to be viewed as another, and perhaps more physiological, form of dopamine-replacement therapy, and by no mean can it be publicized as a cure for PD, in which Lewy pathology extends far beyond the *S. nigra pars compacta* into many brain structures. The basic idea involves: (i) preparing dopamine-producing neurons *in vitro*, and (ii) implanting them into the brain of PD patients. Initial attempts of cell replacement therapy in PD used chromaffin cell-containing adrenal medullary autologous transplants following the report of two apparently spectacularly successful cases (Madrazo et al., 1987). Somewhat disproportionate enthusiasm lead to apply this approach to hundreds of patients worldwide in poorly controlled studies, until it became apparent that grafts failed to survive and to produce reliable, sustained and significant clinical benefits (Goetz et al., 1991). The procedure was abandoned in the early 1990s and researchers, after initial successful experiments in neurotoxin-induced animal models of PD, started to use dopaminergic neuron-containing foetal mesencephalic tissue for allogenic grafting in humans. Pioneered by the Lund group (Lindvall et al., 1990), this approach was assessed through a limited number of small, open-label studies conducted by independent groups (Hagell et al., 1999; Hauser et al., 1999; Brundin et al., 2000; Mendez et al., 2000; Nguyen et al., 2000; Cochen et al., 2003), each using different in-house protocols. These studies have shown a consistent motor improvement, a decrease of total daily doses of levodopa and a massive increase of ^{18}F-fluorodopa tracer of 30–107%, providing evidence that transplanted fetal mesencephalic tissue can survive, integrate and function over a long period of time in the human striatum. It was already apparent, however, that clinical benefit was highly variable from one individual to another. Following these encouraging results, two independent American, placebo-controlled (whereby a burr hole was drilled in the skull but no tissue was implanted) studies were initiated in the mid-1990s, the results of which were published in the early 2000s (Freed et al., 2001; Olanow et al., 2003). In both studies, the placebo group received a sham surgery whereby. In the Denver–New York study (Freed et al., 2001), 40 advanced PD patients were included and followed for 12 months postoperatively using various clinical scales and ^{18}F-fluorodopa PET scans. Twenty, non-immunosuppressed patients received a bilateral transplantation of fetal nigral tissue obtained from a total of 4 donors per patient. As a whole, the group of transplanted patients improved minimally, younger patients (<60 years) performing slightly better than older ones postoperatively. Conversely, there was a significant increase of the radiotracer uptake on postoperative PET scans of about 40%, which was further attested by post-mortem analysis of two patients who died of unrelated causes in the course of the study. In both cases, abundant transplanted dopamine neurons were found with a substantial fiber outgrowth, confirming a very satisfactory graft survival and function. Finally, the study was complicated by the unexpected appearance of levodopa-unrelated dystonia and dyskinesia in 15% of patients, which was attributed to a local excess production of dopamine by the transplant. In the New York–Chicago–Tampa trial (Olanow et al., 2003), a total of 34 patients were included. The study design was relatively similar with the notable exceptions that patients were immunosuppressed with cyclosporine for 6 months, follow-up extended over a 2-year period and the transplantation group ($N = 23$) was divided into subjects receiving tissue from 2 or 8 donors. In this study, authors observed a minimal benefit in the 8-donor group, whereas patients deteriorated in the 2-donor group. Overall, there was no significant treatment effect and, unlike the previous study, there was no age effect upon outcome. In fact, the major complication of this trial was a very high prevalence (57%) of off-medication dyskinesia that was so disabling in some patients that they required a subsequent pallidotomy to manage this adverse event. Again this poor clinical benefit was somewhat paradoxically balanced by a robust survival of dopaminergic neurons, as assessed by PET scans and autopsy data. In all published studies, various factors have been associated with the positive and negative outcomes of fetal cell transplantation in PD patients (Astradsson et al., 2008; Deierborg et al., 2008; Morizane et al., 2008). However, many confounding factors together with only a few randomized studies prevent pinpointing a single key factor to explain a positive or negative outcome.

Despite these discouraging results, most investigators in the field keep thinking that cell replacement therapy has a potential in PD (Dunnett et al., 2001; Winkler et al., 2005). While fetal tissue as a cell-based strategy has been virtually abandoned (Astradsson et al., 2008; Deierborg et al., 2008; Morizane et al., 2008), implantation of dopaminergic derivatives from PSC has now become a particularly interesting alternative to the use of fetal tissue.

5. Major challenges for clinical implementation of pluripotent stem cell-based therapy: example of Parkinson's disease

5.1. Control of differentiation into target cells

PSC have attracted significant attention due to their dual ability to self-renew and to differentiate into any cell type of the body. This makes them excellent candidates for cell- and tissue-replacement therapies, as well as for the basic scientific. However, most available PSC to date have been directly or indirectly exposed to animal material during their derivation and/or propagation *in vitro*. Although there are efforts to use the currently available PSC for clinical trials, such xeno-contaminated cells are generally judged unsuitable for transplantation because of the risk of zoonosis transmitted by animal pathogens and the potential activation of animal retroviruses, not to mention the possibility of immune rejection. For therapeutic purposes, all steps for the production of ESC must avoid the use of animal components, i.e., culture systems using animal-free derivation methods, animal-free culture media, and animal-free substrates should be established to create and propagate clinical-grade ESC lines. Furthermore, driven by their potential for biomedical research and the ease with which they can be generated, many researchers are currently investigating iPSC. Created by reprogramming differentiated adult cells by genetic modification with 2 sets of reprogramming genes, iPSC seem to have regained an embryonic 'stemness' that may allow them to become any type of cell in the body. For the iPSC approach to be clinically relevant, even though the rejection problem is likely to be solved, several challenges remain to be addressed, including minimizing the risks and drawbacks associated with the current method of induction such as genetic modification of the cells with retroviral vectors, avoiding oncogenes among the four reprogramming genes, and improving the low efficiency and slow kinetics of induction.

Although *in vitro* differentiation from PSC to dopaminergic cells is possible, the efficiency of such methods is limited and a number of cellular by-products are generated. The key challenge in PSC cell differentiation is to obtain a maximal number of dopaminergic progenitor cells under clinical-grade conditions while limiting the proportion of other cell types to a minimum at the time of transplantation. Available methods generate high heterogeneity of the cell preparations. The reason for this is not clear. However, there are a number of hypotheses: (i) from PSC to dopaminergic progenitors, cell divisions and differentiation are mixed together in a very complex process. At cell division some parental stem cells are known to produce one daughter stem cell and one daughter cell eventually differentiating into a neural progenitor. This phenomenon is called *asymmetric division*. Thus, after repeated asymmetric cell divisions *in vitro*, various phenotypic subpopulations emerge, including stem cells and differentiated cells (Takagi et al., 2005); PSC cannot start or stop their division and differentiation at the same time. This phenomenon is called *anisochronicity*. Thus, at each defined time point, cultures are a continuum of undifferentiated up to highly mature cells; (ii) neural progenitor cells differentiate into both neurons and glial cells. Thus, astrocytes, neurons and oligodendrocytes are equally present in the culture. Furthermore, heterogeneity is increased because neurons may mature into numerous different subpopulations depending on the type of neurotransmitter synthesized and released; (iii) the dopaminergic is only one of many catecholaminergic phenotypes of a group of cells sharing cellular and biochemical characteristics. Thus, several subpopulations may arise within the dopaminergic lineage: dopaminergic, adrenergic, noradrenergic. As a consequence of such heterogeneity, multiple cell sub-populations are present within a dopaminergic-like population: stem cells, intermediate cells, differentiated cells, etc. The most suitable cells for PD transplantation are dopaminergic progenitors giving rise to dopaminergic neurons of the A9 type, which is with a nigro-striatal phenotype. However, such dopaminergic cells with a therapeutic potential are likely to be mixed with various other rather unwanted cell subpopulations.

Reported protocols for PSC differentiation include the use of non-clinical grade reagents, cell culture supplements that are non-chemically defined or products obtained from animals. Furthermore, most PSC lines used in research originate from co-culture with murine feeder cells and animal sera. Such factors are not compatible with good manufacturing practice and thus, clinical use of PSC is still limited. However, several serum-replacement strategies have been developed to avoid animal products in the differentiation

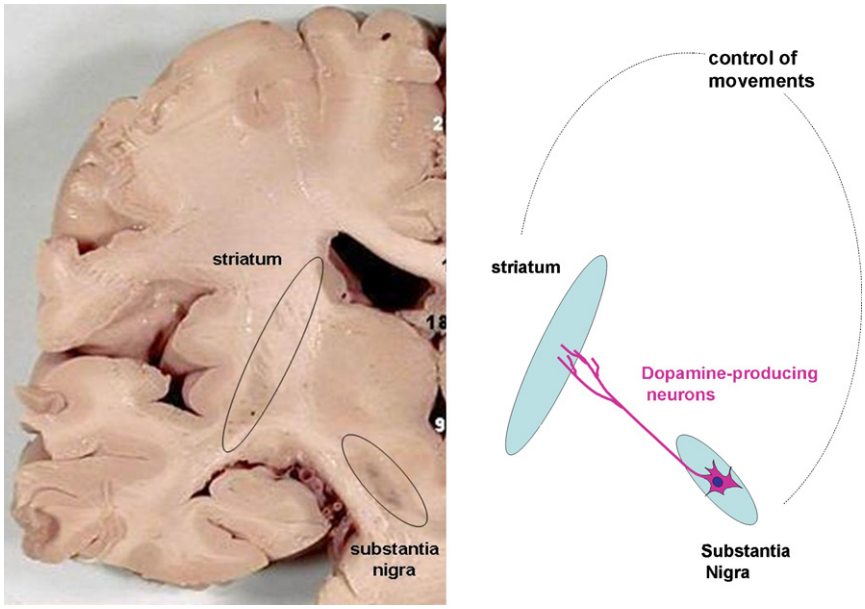

Fig. 2. Site of cell injection for PD. Cell bodies of dopaminergic neurons are located in the *S. nigra*, but axons extend to the striatum where the dopamine-secreting nerve endings need to act. Thus, most animal and human studies on PD cell therapy have injected cells into the *Striatum*. Cell injection into the *S. nigra* might reconstitute a more physiological situation; however it appears that very few axons derived from injected cells are able to extend correctly into the *Striatum*.

protocols (Koivisto et al., 2004) and some attempts have been made to develop more defined, animal product-free PSC maintenance media (Lei et al., 2007; Mallon et al., 2006; Unger et al., 2008a,b). A number of more defined culture media have been developed that require fewer animal-derived components. However none of the currently available formulations are able to maintain PSC in an undifferentiated pluripotent state for an extended period in the way that traditional non-defined formulations do.

5.2. Cell delivery

The basics of cell delivery for PSC therapy of PD are simple, namely stereotaxic injection into the brain. Yet, several important issues are not yet solved (Dunnett et al., 2001; Winkler et al., 2005):

(i) How many cells need to be injected, and should there be only one or multiple injection sites? Also, what is the optimal medium in which cells should be suspended?
(ii) Should cells be injected as a single cell suspension or rather as aggregates?
(iii) What is the optimal site of injection? Dopaminergic neurons that degenerate in PD have their cell body in the *S. nigra*, but their dopamine-secreting terminals extend into the *Striatum*. Cell grafting into the *Striatum* is not physiological; but local dopamine delivery is assured (Fig. 2). In the case of fetal dopaminergic neurons, injection into the *Striatum* has been used in almost all studies. Grafts into the *S. nigra* would be most physiological, but would require axon outgrowth into the *Striatum*. Nigro-striatal axons constitute the nigro-striatal bundle coursing in the medial forebrain bundle (Gerfen et al., 1982). Early studies based on human or mouse foetal progenitors engraftment in lesioned *Striatum* (Wictorin et al., 1990a,b, 1991; Englund et al., 2002; Kelly et al., 2007) have reported the presence of graft-derived axons in the A9 region, their incoming pathway being the internal capsule and cerebral peduncle, structures that extend from the striatum (internal capsule) to the *S. nigra* (cerebral peduncle). Thus axons from cells grafted in the Striatum are able to travel long distances and to reach the A9 region. Even more interesting is the observation that fetal ventral mesencephalic cells grafted in the *S. nigra* are able to grow axons reaching the *Striatum*. When associated with striatal grafting of the same cell type, A9 grafts improve behavioural recovery in 6OH-DOPA treated rats, the physiological recovery being better than with striatal grafts only (Mendez et al., 2000). In human, double grafting of foetal dopamine cells in the *Striatum* and in the *S. nigra* indeed improves Parkinson's Disease Rating Scale (Mendez et al., 2002) and grafted neurons were able to survive up to 4 years in the *S. nigra* (Mendez et al., 2005). Thus as a long-term strategy the *S. nigra* might be a more suitable target for physiological restoration of the nigro-striatal pathway, provided that solutions can be found to assure massive, coherent axon growth towards the *Striatum* and, ultimately, correct synaptic integration in the extra-pyramidal motor system.

5.3. Avoidance of tumors

For PSC-derived cells to become applicable cell therapy, potentially tumorigenic cells (undifferentiated PSC and rapidly proliferating cells) must be eliminated prior to grafting. To date, no published method has fulfilled both of these criteria.

Because PSC are pluripotent, they are potentially tumorigenic following implantation into a tissue (Blum and Benvenisty, 2008). So far, PSC-derived cells have been used for *in vivo* cell transplantation in numerous animal experiments and a few clinical studies. Typically, undifferentiated PSC remain within differentiated PSC-derived cell populations and may result in a high incidence of tumor/teratoma formation, if transplanted. Tumors may derive from many kinds of dividing cells including neural precursor cells and undifferentiated PSC, while teratoma is due to spontaneous proliferation/differentiation of residual undifferentiated PSC after transplantation (that could originates from asymmetric divisions or PSC that did not start or delayed their differentiation program). Both tumor and teratoma formation have been observed *in vivo* following cell transplantation. Therefore, the tumorigenic properties of PSC are of great concern and important for all future transplantation. Prolonged *in vitro* differentiation is supposed to be critical to decrease the incidence of teratomas. At the same time, excessive maturation of dopaminergic progenitors in culture prior to grafting is known to impair their stability for manipulation and implantation. One other factor that could contribute to tumor formation is the occurrence of chromosomal aberrations that are common in ESC following long-term maintenance in culture (Baker et al., 2007; Herszfeld et al., 2006). Chromosomal alterations in ESC were found to occur on 17q, 12 and X (Baker et al., 2007; Draper et al., 2004) and could contribute to cell transformation.

5.4. Control of immune rejection

The origin of ESC in transplantation of PSC is a critical point. The use of genetically unrelated donors of ESC would involve the major hurdle of the immune response towards the transplanted cells. Several barriers have to be considered. The blood group barrier could lead to anti-ABO antibody mediated rejection that could be avoided by transplantation of ABO compatible PSC. Major Histocompatibility Complex (MHC) class I and II antigens are master triggers of robust immunological rejection of grafts and due to the polymorphism of MHC antigen, MHC identical transplantation is not realistic. Finally, the expression of minor histocompatibility antigen by ESC, even not as immunogenic as MHC antigens, are sufficient to drive the immune system to reject the graft (Simpson et al., 2001).

In addition to the allogenic reaction that could be expected in such transplantation, products of animal origin used in the differentiation protocols have also been proposed to amplify the risk of xenogenic antigens inclusion (immunogenic non-human sialoproteins), which in turn increase rejection by the recipient (Martin et al., 2005). Multiple steps have been implemented to minimize the amount of animal components during the differentiation process (Joannides et al., 2007; Nat et al., 2007; Schulz et al., 2004; Yan et al., 2005) including the replacement of bovine serum in the medium (Koivisto et al., 2004).

Nevertheless, the potential importance of immune rejection process remains a subject of intense debate. Several experimental studies showed little concern for potential cellular immune problems associated with ESC transplantation. Undifferentiated ESC express low levels of HLA class I (MHC-I), which is up-regulated by IFN-γ stimulation or after differentiation into embryoid bodies as well as teratoma formation (Drukker et al., 2002; Grinnemo et al., 2006; Li et al., 2008). MHC class II (MHC-II) and co-stimulatory molecules, however, have not been found (or at low levels) under these circumstances, suggesting that ESC lack the two important prerequisites to function as professional antigen presenting cells. Spontaneous differentiation of ESC into embryoid bodies or teratoma causes an increase in MHC-I (Drukker et al., 2002). However, the absolute expression levels were observed to be under those of other somatic cells analyzed. Conflicting data were also observed in studies analyzing allogeneic T cell proliferation stimulated by ESC in mixed lymphocyte reaction studies. ESC have been shown to induce similar T cell proliferation levels as cultured human fibroblasts (Grinnemo et al., 2006). More specifically for fetal neural progenitor cells, the expression of MHC-I and -II, but not the co-stimulatory proteins CD40, CD80 and CD86, increased significantly after IFN-

γ stimulation, but peripheral lymphocytes were unresponsive in MLR, suggesting their low immunogenicity despite HLA incompatibility and high HLA expression (Odeberg et al., 2005).

As MHC-I molecules are ligands for some inhibitory receptors of NK cells, the absence of expression or the low level of MHC-I on human ESC made such cells good target for NK cell killing. However, previous study demonstrated that irrespectively of the differentiation status of the cells and the expression levels of MHC-I *in vitro*, ESCs were not killed by NK cells, even if inhibitory HLA-G molecules are expressed (Drukker et al., 2002). Therefore, it has been suggested that ESC and their derivatives were not killed by NK cells because of lack of recognition rather than inhibition. Using two experimental cell types, the leukocyte response toward ESC was investigated *in vivo* in a mouse model. Immunodeficient mice corrected with peripheral leukocytes from human origin showed only a minimal immune response toward undifferentiated as well as differentiated ESCs probably due to a low immunostimulatory potential (Drukker et al., 2006). Small animal studies are encouraging with regards to the low level of immunogenicity of ESC. However, we have learned from many transplantation models that what can be achieved in rodent models in term of tolerance or immunosupression is too often not available for large animal and human studies. This can explained why despite the apparent low level of immunogenicity of foetal or ESC-derived neural progenitor cells, most of the clinical trials in humans using fetal material included empirically immunosuppressive drugs such as cyclosporine (Freed et al., 2001; Olanow et al., 2003). However, studies currently investigating the rationale of immunosuppressant use in stem cell transplantation are lacking.

5.5. Prevention of infections

Differentiated PSC should be regarded as a medicinal product and thus underlie the pharmaceutical legislation of the European commission (http://www.ec.europa.eu/enterprise/pharmaceuticals/eudralex). Specifically volume 4 about Good manufacturing practice (GMP) controls quality issues from starting materials until the final product. The different drug categories are addressed separately and include biological products. Specific recommendations for PSC are soon to be released. The legally binding GMP guidelines direct process documentation, cleanliness of the premises as well as appropriate quality controls in the production process. The main concerns about PSC-derived "products" are that they provide a good substrate for growth of microbial contaminants and that they cannot be terminally sterilised. Furthermore, PSC may be exposed to animal retroviruses during their differentiation. Thus, all handling must be carried out aseptically and microbiological controls exceed standard procedures.

Three major infection problems may be distinguished: (i) infection of the biological material to be transplanted; (ii) contamination during elaboration and handling of the transplant; (iii) post-transplant infection of the recipient due to immunosuppression and/or the surgical procedure

(i) Infection of the biological material to be implanted. The typical example for this problem is the transplantation of CMV-positive bone marrow or a solid organ into a CMV-negative recipient (Akalin et al., 2003; Singh et al., 2003). Presently, there is no evidence for persistent infection of ESC or iPSC, however more systematic research on this topic is needed and, before clinical applications, all clinic-compatible lines will have to be screened for a large number of potential pathogens. Two situations deserve particular attention: (1) Mycoplasma infection is commonly encountered in cell cultures (Uphoff and Drexler, 2005). Thus, contamination with this pathogen may arise at any time in the cell differentiation process and, apart from continuous routine-screening, additional screening directly before transplantation is advisable (Cobo et al., 2007). (2) The second problem of interest is contamination and potential reactivation of endogenous retroviruses. Animal feeder cells have been shown containing retroviral particles (Cobo et al., 2008). HIV and HTLV I can be transferred by transplantation of bone marrow or solid organs (Atkinson et al., 1987; Gonzalez-Perez et al., 2003; Simonds et al., 1992; Soyama et al., 2008). Thus, a close eye on this risk should be kept in the context of PSC transplantation. Given the fact that PSC may have been exposed to animal feeder cells or to animal-derived differentiation factors, the issue of animal retrovirus transmission is serious. Avoiding use of animal feeder cells and animal-derived reagents in PSC processing is essential.

(ii) Contamination during elaboration and handling of the implant. The laboratory conditions are designed to provide optimal growth for PSC. Such techniques provide optimal growth conditions not only for stem cells but for extraneous microbial contaminants as well. Because cells withstand a wide range of purification techniques, all processing must be conducted under strict aseptic conditions. The differentiation process until dopaminergic progenitor cells can be injected in the patient is completely different from other types of transplantation. While, for example, preparation of haematopoietic stem cells for bone marrow transplantation is a quite rapid procedure, the preparation of dopaminergic neurons for PSC transplantation is expected to take at least 5 weeks. In particular bacterial pathogens from the environment or from laboratory workers may contaminate cell cultures at any time. Thus, routine microbiological screening of the environment and the cell cultures need to be performed.

(iii) Post-implant infection of the recipient due to the surgical procedure and/or immunosuppression. In the case of PSC therapy of PD, the surgical procedure is relatively simple (stereotaxic injection). Nevertheless, occasional introduction of pathogens by the procedure cannot be excluded. Gram-positives and in particular *Staphylococcus aureus* may cause dangerous surgical site infections within days or weeks after the intervention. Despite the expected low immunogenicity of PSC after transplantation, some sort of immunosuppression has likely to be established, at least in the first phases of clinical trials. The extent of such immunosuppression is not known yet. However, opportunistic infections with pathogens such as *Nocardia*, *Listeria*, *Pneumocystis* or *Cryptooccci*, or reactivation of CMV cannot be excluded at this point.

5.6. Design of clinical studies

The clinical protocol of the first studies in humans using PSC should be carefully designed so as to minimize unforeseen patients-related factors that may have a negative impact on post-transplantation outcome, as suggested in the foetal nigral transplantation trials (Winkler et al., 2005). Notably, emphasis should be placed on patient selection and stringent inclusion and exclusion criteria should be established, in light of what has been learned in the past. The profile of the ideal candidates should be separately defined for phase I to phase III studies, as criteria may change from one study type to another, although, conversely, a homogeneous population is probably desired for reliable conclusions to be achieved. For example, a key inclusion criterion involves a firm diagnosis of clinically typical, levodopa-responsive PD, based on universally accepted diagnostic criteria such as those proposed by the United Kingdom PD Brain Bank, and on relevant ancillary tests such as olfactometry and dopamine-oriented metabolic neuroimaging. Particular effort should be made to exclude atypical

parkinsonian syndromes, because it has been well demonstrated that such patients do not, or only poorly respond to dopamine-replacement therapy. Other characteristics should be carefully considered including age at the time of the procedure, disease duration, disease severity and presence of motor fluctuations and dyskinesia. In general, it can be inferred that motor symptoms that do not respond to levodopa, such as gait impairment, postural instability and dysphagia, or non-motor symptoms such as sleep disturbances, autonomic dysfunction, depression and cognitive decline, may be equally resistant to PSC-derived dopaminergic neuron grafting. Therefore, it is probably undesirable to include aged patients with advanced PD in these trials. In addition to concerns regarding informed consent in patients who will often suffer some degree of cognitive impairment, potential motor benefit may be confounded by too many non-motor aspects of the condition, as already demonstrated for subthalamic nucleus deep brain stimulation (Russmann et al., 2004).

The design of the first clinical studies will be crucial and, at the present time, there is no clear consensus how this should be done. The first question is whether a first clinical study will be a true phase I trial, exclusively concerned with safety and not with efficacy. Or should the first study be, as generally done in the field of cell therapy, a mixed study examining safety and efficacy simultaneously. Purely scientific considerations might favour the first possibility. While the risk/benefit ratio of such trials clearly seems to require that a phase I study be conducted on patients rather than healthy volunteers, further ethical analysis will be needed to evaluate whether separating or combining phases I and II is preferable.

Finally, the read-out will be of major importance to precisely determine the clinical outcome. A combination of clinical scales and neuroimaging studies represents the most appropriate approach. With respect to neuroimaging studies, ^{18}F-fluoro-dopa PET scan might presently appears as the most promising tool to assess graft survival and function (Brooks, 2004).

5.7. Ethical an legal considerations

Several ethical issues are associated with the future clinical application of PSC. These include ethical questions related to the derivation of ESC from human early embryos, experimental protocols in animals with a special emphasis on research involving non-human primates and the ethical prerequisites to initiate clinical studies in human subjects.

The derivation of ESC has been thoroughly researched and it is now subject to detailed legal regulations in many countries (e.g. EudraLex, 2005. The Rules Governing Medicinal Products in the European Union, vol. 4: Good Manufacturing Practice. Part I and II; EudraLex, 2007. The Rules Governing Medicinal Products in the European Union, vol. 4: Good Manufacturing Practice. Manufacture of Biological Medicinal Products for Human Use. Draft Annex 2: 1–25; DH, 2001. A code of practice for tissue banks providing tissues of human origin for therapeutic purposes). Although issues surrounding the destruction of human blastocysts in the context of ESC research continue to inspire scholarly reflexion (Chan and Harris, 2008; Baertschi et al., in press), many remaining ethical questions do not directly concern the ethical standing of the human (Sugarman, 2008), such as the treatment of gamete and/or embryo donors, the timing and procedure for informed consent of couples who might consider donating spare embryos to research, and the management of possible conflicts of interest between the different professionals involved. These issues require specific ethical reflection since they are not completely covered by legal rules, even in countries which have legislated human embryonic stem cell research extensively.

This is the case in Switzerland, since the Stem Cell Research Act of 2005 makes it legal to derive embryonic stem cells from spare embryos remaining after infertility treatment and to do research on them (http://www.admin.ch/ch/f/rs/8/810.31.fr.pdf, in French). However, there are still compatibility problems with earlier, more conservative legislation that has not yet been amended accordingly. As a result, many practical issues remain, especially as regards the modalities of consent by couples considering donating embryos for research

The question of experimentation on non-human primates before clinical application of PSC is an important ethical issue. To date, only one study reported the effect of PSC transplantation in monkeys. The ethics of animal use and animal experimentation have of course been a matter of controversy for a long time. On the one hand, the concept of “speciesism” is central to animal rights movements’ attempts to claim for the fight against ‘discrimination’ of sentient animals the same moral high ground as for struggles against racism and sexism. On the other hand, for any given biological question, a short “translational distance” between animal and human is what makes an animal model valuable scientifically as well as for anticipating risks for future human subjects. Yet the closer an animal model is to human functioning as regards cognitive abilities, the more troubling the ethical question of animal use becomes.

Animal experimentation has long been a topic of ardent political debate in Switzerland, since the Swiss voters have been called repeatedly to express themselves on this issue. The recently revised (2008) Animal Protection Act (http://www.admin.ch/ch/f/as/2008/2965.pdf, in French) raises controversial questions as to the permissibility of primate studies, because the purpose of the law is said “to protect the dignity and welfare of animals” (Art. 1). Introducing the language of ‘dignity’ in this context raises many perplexing questions as to its practical implications, if any. A test case concerning experimentation on macaques is presently reviewed in the court system (Abbott, 2008), but no final sentence has been reached at the time of writing.

A significant effort of bioethical research and conceptual clarification is required in anticipation of the first protocols involving human subjects. Although there is a detailed regulatory environment for human subject research generally, described in documents such as the declaration of Helsinki or the ICH tripartite guidelines, that framework is to a large extent geared towards testing new molecules and not always applicable to cell therapy. Furthermore, the main ethical issues lie “upstream”, as it were, in the question of when it is ethical to begin with human experimentation of stem cell-based treatments. Simply transferring onto cell therapy the well worked-out conceptual frameworks applied in drug development, such as the principles of Good Laboratory Practice (GLP) required to apply for a New Drug Application with the US FDA, is unlikely to suffice. Neither is Switzerland’s planned revision of its legislation on research with human subjects likely to regulate such ‘technical’ details. Aspects currently not resolved by regulations and codes of research ethics include how best to minimize risks in very early human trials, selecting subjects fairly when targeting a disease affecting cognition, and making research participation sufficiently understandable for informed consent in such a setting. Defining when risks are sufficiently understood and controllable to go ahead with human studies will also require the development of new benchmarks in close collaboration between ethicists and both pre-clinical and clinical researchers.

6. Conclusion

Cell replacement therapy of PD: science fiction or *Hannibal ante portas*? To the present understanding it is neither one nor the other. There is clear and advanced evidence in the literature suggesting that PSC may become a useful weapon in the limited arsenal of

treatment opportunities for PD. However, there are still many questions to be addressed and problems being solved before the first patient can be successfully treated with PSC. Two mistakes to be avoided under all circumstances are as follows: (i) raising unrealistic public expectations of the rapid disposition of a PSC replacement therapy; (ii) treating patients too early without having established all necessary protocols and safety precautions. The field of stem cell therapy is rapidly growing and substantial advances can be expected over the next few years.

Conflicts of interest

The authors of this article declare that they have no conflicts of interest.

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