Review

Critical issues of clinical human embryonic stem cell therapy for brain repair

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Embryonic stem cells (ESCs) provide hope as a potential regenerative therapy for neurological conditions such as Parkinson's disease and spinal cord injury. Currently, ESC-based nervous system repair faces several problems. One major hurdle is related to problems in generating large and defined populations of the desired types of neurons from human ESCs (hESCs). Moreover, survival of grafted hESC-derived cells has varied and functional recovery in recipient animals has often been disappointing. Importantly, in clinical trials, adverse effects after surgery, including tumors or vigorous immune reactions, must be avoided. Here we highlight attempts to overcome these hurdles with hESCs intended for central nervous system repair. We focus on hESC-derived dopamine-producing neurons that can be grafted in Parkinson's disease and identify critical experiments that need to be conducted before clinical trials can occur.

Introduction

Neuronal cell death is a core feature of both acute central nervous system (CNS) insults (e.g. stroke and trauma) and several neurodegenerative disorders, including Alzheimer's, Parkinson's (PD) and Huntington's diseases. Cell replacement therapy, therefore, is potentially an attractive strategy in these conditions. In PD, grafts of mesencephalic tissue, obtained from aborted embryos/fetuses, can dramatically reduce motor symptoms. Unfortunately, the dramatic effects seen in patients in open-label trials did not translate into the same degree of success in two double-blind, placebo-controlled studies, which both reported that nigral transplants did not produce significant benefit in the primary outcome measures [1,2]. Owing to large variation in outcome between patients, shortage of donor cells and adverse effects such as graft-induced dyskinesias in some patients [3,4], it is unlikely that neural transplantation using aborted fetal tissue will ever become a routine treatment for PD. An alternate source of dopamine-producing neurons that will allow for greater reproducibility and carries less risk of side effects is definitely needed. Human embryonic stem cells (hESCs) are strong candidates to fulfill this role, but as we describe in this article they are associated with their own set of problems.

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What are the major challenges facing the use of hESCs for clinical cell therapy? The first is to develop cell-culture protocols that generate relatively defined and large numbers of transplantable cells. The second is to obtain adequate cell survival and functionality of grafted cells after intracerebral transplantation. Third, potential adverse effects of transplanted hESC-derived cells, such as tumor formation, must be avoided. Finally, the risk for immune rejection of the grafted cells must be eliminated. In this review, we discuss these issues and provide tentative suggestions for solutions. We focus on cell therapy in PD as an example where the potential of hESC-derived cells might be tested. We believe that the development of hESCs as a source of donor tissue for grafting in PD is likely to provide vital information that will be useful for cell therapy in several other neurological conditions.

Generating sufficient number of hESC-derived cells for transplantation

One fundamental challenge facing all forms of stem cell-based therapy is that large quantities of a specific cell type must be generated, preferably without contamination of other cells that could be detrimental. Defining conditions under which a small proportion of stem cells differentiates into a desired cell type is relatively easy, but it is more difficult to devise strategies where the vast majority develops into the desired cell type. Mouse ESCs (mESCs) have been used both to study basic mechanisms of neural differentiation and to develop culture protocols that yield specific cells needed for transplantation. In principle, three types of approaches are used: (i) co-culture with feeder cells; (ii) addition of soluble growth and neurotrophic factors; and (iii) genetic manipulations of ESCs. In many cases, similar protocols have been adapted and used with hESCs (Table 1).

Culture protocols based on growing ESCs together with specific feeder cells have been widely used and similar proportions of tyrosine hydroxylase (TH)-positive neurons have been obtained from mouse and human ESCs [5–9]. Approximately 16% of mESCs became TH positive when co-cultured with stromal cell feeder cells [7]. The feeder cell-derived molecules that govern the differentiation of ESCs have still not been identified, despite several experiments dedicated to this task. Therefore, it has not yet been possible to replace the feeders with synthetic substrates or specific additions to the culture media. The feeder cells

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Table 1. Protocols used to differentiate ESCs from mouse and human sources into dopaminergic neurons

ESC sources	Key features of differentiation methods *in vitro*			TH^+ neurons *in vitro*	Graft survival	Refs
	Culturing conditions	Soluble factors used	Genetic manipulation			
mESC	Grown with PA6 mouse stromal cells	–	–	16% of total cells	3% of total number of implanted cells	[7]
mESC	Grown as EBs	bFGF, SHH, FGF-8, AA	–	5% of total cells (7% of 72% TuJ^+ cells)	ND	[11]
mESC	Grown as EBs	bFGF, SHH, FGF-8	*Nurr1* overexpression	78% of total cells	4% of total implanted cells	[18]
mESC	Grown as EBs	bFGF, FGF8, SHH	*Pitx3* or *Nurr1* overexpression	25% of TuJ^+ cells	3.4% of total implanted cells	[17]
mESC	Grown with MS5 mouse stromal cells	SHH, FGF-8, bFGF, BDNF, AA	–	ND	10%–20% of total implanted cells	[8]
mESC	Grown with PA6 mouse stromal cells	SHH, FGF-8, AA	*Nurr1* overexpression	56% of total cells (90% of 62% TuJ^+ cells)	312 TH^+ neurons/mm^3	[15]
mESC	–	bFGF, SHH, FGF-8	*Lmx1a* overexpression	TH^+ cells in 60% colonies	ND	[14]
hESC (BG01)	Grown with PA6 mouse stromal cells	–	–	TH^+ cells in 87% colonies	9 TH^+ cells/section	[24]
hESC (SA002)	Grown with PA6 mouse stromal cells	–	–	7% of total cells	10–50 TH^+ cells/graft	[5]
hESC (H1, H9)	Grown as EBs with human fetal midbrain astrocytes	bFGF, SHH, FGF-8	–	40% of total cells (67% of TuJ^+ cells)	27 000 TH^+ neurons/mm^3	[19]
hESC (BG01, BG03)	Grown as EBs	bFGF, BDNF, GDNF	–	75% of TuJ^+ cells	A few TH^+ cells	[23]
hESC (H9, BG01, HUES7, HUES8)	Grown as EBs; formation of rosettes	bFGF, dbcAM	–	56%–81% of all colonies	ND	[12]
hESC (HES-1)	Grown as spheres	MEF, Noggin, bFGF, EGF	–	0.5% of total cells (29% of TuJ^+ cells)	389 TH^+ cells (0.18% of total grafted cells)	[25]
hESC (H1, H9)	Grown as EBs	bFGF, FGF-8, SHH, BDNF, GDNF, AA	–	31% of total cells	ND	[67]
hESC (H9)	Grown as EBs	bFGF, FGF-8, SHH, Wnt3a, BDNF, GDNF, AA	–	43% of TuJ^+ cells	1273 TH^+ cells/graft	[26]
hESCs (H1, H9, HES-9)	Grown with MS5 mouse stromal cells	SHH, FGF-8, BDNF, GDNF, TGF-β3, dbcAMP, AA	–	19%–39% of total cells (64% of 30%–79% TuJ^+ cells)	ND	[9]
hESC (HSF-6, SNU-hES-3, Miz-hES-1)	Grown with PA6 mouse stromal cells	bFGF, SHH, BDNF, NT-3, FGF-8	–	12% of total cells (41% of 30% TuJ^+ cells)	Few TH^+ cells	[10]
hESC (H7, H9)	Grown with MS5 mouse stromal cells	SHH, FGF8, BDNF, GDNF, TGFβ, AA	–	23% of total cells	160 TH^+ cells/graft	[68]

ND, not determined. For explanation of abbreviations, see main text and the original papers.

typically are not of human origin, which will complicate their inclusion in a clinical-grade culture protocol. They add variability and complexity to a protocol, increase the workload and consequently limit large-scale culture of hESCs. Moreover, culture media to date often contain animal-derived products, which prohibit their use in clinical protocols. Importantly, feeder-free cultures in defined media lacking animal products would give more reproducible culture conditions, facilitate scale-up and permit the clinical use of the resulting hESC-derived cells.

Defined soluble factors have been widely used in differentiating ESCs into neurons alone or in combination with co-cultures [5,6,10–13]. Several factors have been tried in differentiation protocols, such as fibroblast growth factor (FGF) 2, FGF-8, FGF-20, glial cell line-derived neurotrophic factor (GDNF), neurturin, brain-derived neurotrophic factor (BDNF), sonic hedgehog (SHH) and ascorbic acid (AA). Use of defined chemicals and growth factors provides a "cleaner" differentiation protocol than feeder cells and might therefore be comparatively easier to apply in clinical trials. In the context of dopamine neuron differentiation, however, the signaling pathways used by these factors are not well understood. Consequently, the protocols have developed through empirical studies and often they require several weeks of differentiation *in vitro*.

Genetic manipulation is another strategy to improve the rate of differentiation of dopaminergic neurons from ESCs that has been tested extensively. Specific genes, typically transcription factors important for dopaminergic neuron specification during normal development, have been overexpressed in mESCs [14–17]. For example, expression of *Nurr1* or *Lmx1a* substantially increases differentiation of mESCs into dopaminergic neurons [14,18]. Unfortunately, the genetic manipulation of hESCs appears to be less effective. Furthermore, genetic manipulation in hESCs might create additional safety risks in clinical applications.

The best differentiation protocol for mESCs to date is the combination of the above-mentioned three methods: feeder cells, growth factors and genetic engineering [15]. Using this particular method, ~90% of mESC-derived β-III

tubulin-positive cells develop into TH-positive neurons. The yield of dopamine neurons has been almost as high when using similar types of strategies to differentiate hESCs [9,19]. From a clinical perspective, however, it is desirable that the culture protocols are feeder free, xeno free and do not involve viral vectors.

Survival of hESC-derived neurons after transplantation needs to be improved

As expected, good cell survival is essential for grafts to be effective in clinical cell transplantation in neurological diseases [20]. This is in good agreement with studies in experimental animals. Thus, neural cells derived from mESCs survive transplantation in rodent models of PD and support behavioral recovery [7,18,21]. Similarly, dopamine-producing neurons derived from monkey ESCs survive grafting into monkeys subjected to lesions of the dopamine system [6,22]. By contrast, whereas human ESC-derived neurons survive transplantation to immunosuppressed rodents, their effects on neurological deficits in recipient animals are less encouraging [5,19,23]. Typically, hESC-derived grafts into the brain contain relatively few surviving dopaminergic neurons and frequently generate tumors [5,10,23,24]. In most reports, when 100 000–400 000 hESC-derived cells are transplanted into the striatum, less than 300 TH-positive neurons survive [5,23,25]. This is fewer than the number necessary to reinstate dopamine neurotransmission in rodents with nigrostriatal lesions, and therefore such grafts do not exert clear effects on motor deficits [5].

A recent study demonstrated that five out of six grafted animals gradually develop functional recovery in amphetamine-induced rotation tests. About 1300 TH-positive cells survived in each graft [26]. In another recent study, a very large number of hESC-derived TH-positive neurons survived grafting [19]. The authors suggested that the implants exerted beneficial effects on the behavior of recipient rats [19]. Because of continued proliferation of implanted cells, resulting in very large transplants and unusual features of the employed rat PD model, it is unclear whether dopamine release from the graft caused the behavioral changes seen after transplantation [27]. Notwithstanding the issues of the functional efficacy of the implants, the study demonstrated that, under certain conditions, large numbers of hESC-derived dopaminergic neurons (~27 000 TH-positive cells/mm^3 of graft tissue) can survive grafting to rodents [19].

Why have several studies failed to demonstrate numerous dopaminergic neurons in grafts derived from hESCs? Two main explanations seem possible. First, in most cases the cells were relatively mature (with long processes, etc.) when harvested from the cultures, and cell death might have been excessive. Grafted dopaminergic neurons obtained from fetal brains are known to survive poorly if their maturation has passed a narrow optimal time window at the time of harvesting [28]. Second, it is possible that the cells actually survive the grafting but cease to express TH and other proteins characteristic of dopamine neurons. Clearly, numerous cells expressing the mature neuronal marker protein NeuN can be found in hESC-derived grafts, but the proportion that is TH immunopositive following the transplantation is low [5,26]. Recently, we observed a similar phenomenon when transplanting human ventral mesencephalic progenitors into the striatum [29]. Prior to grafting, around 20% of the cells expressed TH. Two to 11 weeks after implantation into the adult rat striatum, the grafts contained numerous neuron-like cells, but none of them were TH positive. Parallel *in vitro* experiments, where the cells were switched between different culture conditions, suggested that grafted human TH-expressing neurons do not all die, but instead exhibit unstable TH expression [29]. Thus, instability of the dopaminergic phenotype in hESC-derived neurons might contribute to the low frequency of TH-positive cells seen in intracerebral grafts. Therefore, it is important to address both the survival and the stability of the grafted dopaminergic neurons derived from hESCs. Ideally, the cultured cells should have differentiated into postmitotic dopaminergic neurons with short neuronal processes at the time of grafting. This would minimize the detrimental effects of axotomy during cell harvesting, and thereby potentially enhance TH neuron survival in the grafts. In conclusion, the true capacity of hESC-derived neurons to exert beneficial effects in animal models of PD cannot be fully assessed until large numbers of dopaminergic neurons survive and retain their full phenotype after transplantation.

Avoiding adverse effects after grafting hESC-derived cells to patients

Several adverse events might occur when transplanting hESC-derived cells into the CNS. In the following sections, we briefly describe undesirable events that could take place when hESCs are grafted to the brains of patients. For example, residual undifferentiated hESCs or dividing precursors might continue to proliferate *in vivo* and generate tumors. Chromosomal instability might contribute to chromosomal aberrations during long-term culturing of hESCs [30]. This has unpredictable repercussions that obviously depend on the specific chromosomal changes. Once the cells are grafted, rapidly dividing hESCs with chromosomal changes might outgrow other cells in the transplant and promote tumor formation. Finally, hESC-derived grafts could stimulate an immune reaction in the brain, and this might adversely affect surrounding brain circuitry.

Transplanted hESCs stand a risk of forming tumors

The tumorigenic properties of hESCs restrict their potential usefulness in clinical cell transplantation [31,32]. Within a few weeks following transplantation, transplanted ESCs might form tumors. In some cases, they contain tissues from all three germ layers and thereby constitute teratomas [33,34]. Tumors containing immature cells can still form when the majority of the grafted ESCs have undergone prior *in vitro* differentiation along defined lineages [5,35–37]. Although this is relatively rare [18,38,39], it is a serious risk factor in cell transplantation to the brain. The tumors that form after transplantation of differentiated ESCs are considered to be residual proliferating ESCs or precursors (Figure 1). Regardless of which differentiation protocol is used, hESC-derived cells are

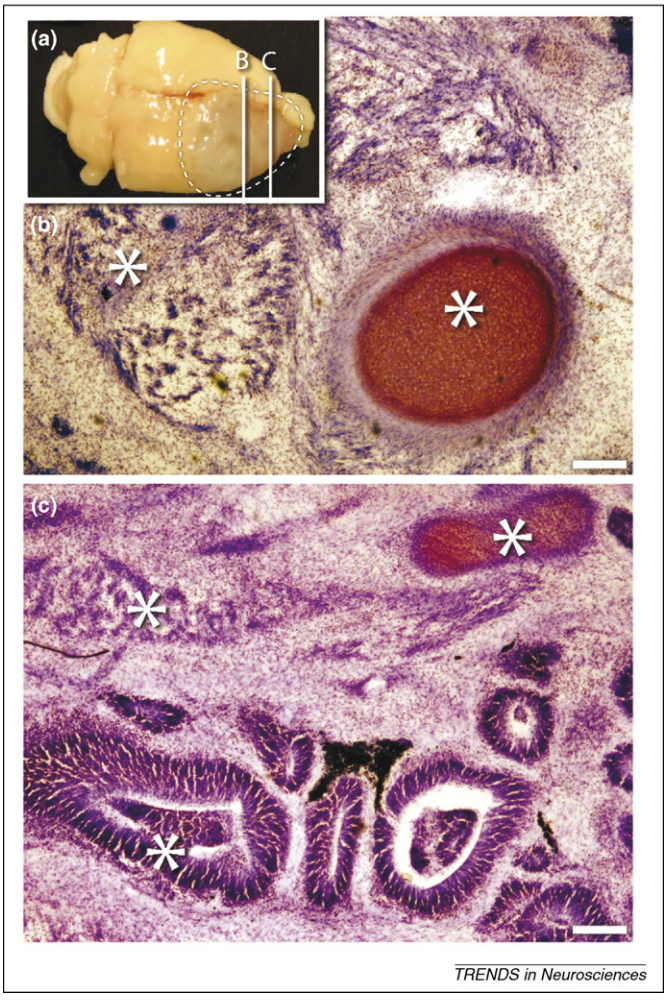

Figure 1. Macroscopic image of a brain showing a teratoma in the grafted site 11 weeks after transplanting 16 day pre-differentiated hESCs. The teratoma is delineated with a dashed line (**a**). White lines indicate the section plans for (**b**) and (**c**). (b) and (c) Microscopic images of coronal sections showing different types of tissue structures reminiscent of teratomas (asterisks). Bars = 400 μm.

clearly heterogeneous unless they are subjected to cell sorting. Therefore, strategies to remove undifferentiated hESCs and progenitors that otherwise might continue to proliferate after transplantation (see Box 1 for details) will be important. In principle, potentially several different methods can be used to achieve this. We demonstrated that prolonged pre-differentiation of hESCs substantially reduces the incidence of tumor formation after grafting [5]. We observed tumors resembling teratomas in rats receiving grafts of cells co-cultured for 16 days on stromal feeder cells. The tumor incidence was reduced when hESCs were co-cultured for 20 days, and we found no tumors in rats receiving transplants of hESCs differentiated for 23 days. However, some cells were still undergoing mitosis in the latter grafts. Therefore, we cannot completely exclude that the grafts would form tumors in the long term after implantation. Nevertheless, the experiment indicates that extensive *in vitro* differentiation before grafting, as expected, reduces the risk of tumor formation. As mentioned earlier, dopamine neurons tend to die if they have undergone excessive differentiation before transplantation. Therefore, although extensive cell differentiation before grafting might reduce the risk of teratomas, it might not be optimal when grafting dopamine neurons.

Another approach might be to knock down or block intracellular signaling pathways that promote proliferation and cell survival. This can prevent further mitosis, and lead to apoptosis or induce differentiation into nondividing cells. Tumor formation can clearly be prevented by genetically manipulating mESCs. Selectively removing *Cripto* expression in mESCs alters the Nodal pathway and reduces tumor formation, which has been demonstrated in a study on $Cripto^{-/-}$ mESCs grafted to the striatum in a rat model of PD [40]. The absence of *Cripto* also promotes differentiation into neurons. In addition, it has been suggested that ESCs can be genetically

Box 1. Strategies for eliminating tumors

Removal of undifferentiated hESCs and proliferating cells from cultures, before transplantation, is potentially a powerful approach to reduce the risk of tumor growth. Here we list several potential strategies.

Prolonged pre-differentiation of ESCs *in vitro*
We have demonstrated that prolonged pre-differentiation of hESCs substantially reduces the incidence of tumor formation after grafting [3]. We observed tumors resembling teratomas in rats grafted with cells co-cultured for 16 days on stromal feeder cells. The tumor incidence was reduced when hESCs were co-cultured for 20 days and completely eliminated when hESCs were differentiated for 23 days. However, this strategy alone is unlikely to achieve complete elimination of tumors, and it might even jeopardize survival of the desired neurons that can mature too much *in vitro* before grafting to the brain.

Knockdown/blocking of intracellular signaling pathways
This might prevent further mitosis, leading to apoptosis or inducing differentiation into nondividing cells. Tumor formation can be prevented by genetically manipulating mESCs. Selectively removing *Cripto* expression in mESCs alters the Nodal pathway and reduces tumor formation, following grafting of $Cripto^{-/-}$ mESCs to the striatum [40]. Moreover, by genetically engineering ESCs to express the herpes simplex virus thymidine kinase (HSV-tk) gene, in the presence of ganciclovir, undifferentiated ESCs can be efficiently eliminated [42].

Enrichment of cells using fluorescent reporter markers and cell sorting
This has been applied to cultures of mESCs. Neuronal precursors expressing a fluorescent reporter gene coupled to Sox1 were positively sorted by using fluorescent activated cell sorting (FACS) and transplanted into the striatum. These grafts did not form tumors by 8 weeks posttransplantation [44]. This strategy, however, is not appropriate for immediate translation to a clinical setting, as it requires genetically modified ESCs.

Selective depletion using conjugated cell-surface antibodies
Technologies such as FACS or magnetic activated cell sorting (MACS) might be employed to deplete dividing cells or residual undifferentiated ESCs. This cell-depletion strategy is advantageous because it does not require modification of the ''desired cell type.''

Pharmacological agents
These have been used to induce apoptosis of residual proliferative mESCs within embryoid bodies (EBs) [37]. Following dissociation of EBs and MACS to remove apoptotic cells, the remaining cells were transplanted into the mouse striatum. They yielded significantly fewer tumors than control grafts (untreated EBs), although tumors were not completely prevented.

In practice, the above-mentioned methods might have to be combined, to achieve safe transplantation of hESC-derived cells.

engineered to express the herpes simplex virus thymidine kinase (HSV-tk) gene [41]. In the presence of ganciclovir, undifferentiated ESCs can be efficiently eliminated *ex vivo* and *in vivo* [42]; this therefore opens the opportunity to use pluripotent genes, such as Oct 3/4 and Nanog-regulated promoter, to control HSV-tk gene expression to specifically ablate the undifferentiated ESCs from cell populations before transplantation.

Removal of undifferentiated hESCs and proliferating cells before grafting is potentially a powerful approach to reduce the risk of tumor growth. Undifferentiated ESCs express unique cell-surface molecules. Upon differentiation, expression of these molecules is downregulated. Therefore, these undesired cells can be depleted by using antibodies against specific cell-surface molecules conjugated with either a fluorophore or a magnetic bead in combination with fluorescent activated cell sorting (FACS) or magnetic activated cell sorting (MACS), respectively. Using the FACS approach on mESCs, the risk for intracerebral tumors is reduced. The overall graft survival, however, is also compromised, indicating that the method needs further fine-tuning [43]. The reverse strategy, that is, enrichment of differentiated cells by cell sorting, has also been applied to cultures of mESCs. Specifically, neuronal precursors expressing a fluorescent reporter gene coupled to *Sox1* were positively sorted and transplanted into the striatum. These grafts did not grow into tumors by 8 weeks posttransplantation [44]. By contrast, grafts of *Sox1*-negative cells developed into tumors in 55% of cases. Although several dividing, Ki67-positive cells were also found in the *Sox1* grafts, none of them expressed SSEA1, a marker for pluripotent ESCs. Long-term studies are required to evaluate whether the proliferating cells continue to divide or become postmitotic and differentiate. The *Sox1* genetically modified cells are useful tools to study factors that influence tumor formation and can be used to investigate the effects of targeting specific types of cells. They are not, however, appropriate for immediate clinical translation because they require genetically modified ESCs that express reporter genes coupled to specific promoters, which has been difficult to create in hESCs. Therefore, negative depletion strategies or other nongenetic modification approaches should be further pursued. In this context, it is clear that FACS is relatively harsh on cells that possess long processes, as do neurons and their maturing precursors. On the other hand, MACS appears to be less damaging to such cells and therefore could prove more useful when sorting cells before transplantation into the nervous system.

A conceptually simple and attractive approach to eliminating residual undifferentiated ESCs from cell cultures is to add pharmacological agents that target the dividing cells. The ceramide analog N-oleoyl serinol (S18) has been used *in vitro* to induce apoptosis of residual proliferative mESCs within embryoid bodies [37]. Following dissociation of the embryoid bodies and MACS sorting to remove apoptotic cells, the remaining cells were transplanted into the mouse striatum. They yielded significantly fewer tumors than control grafts of cells from untreated embryoid bodies, although tumors were not completely prevented. The tumors that formed were almost exclusively nestin positive, indicating that they were of neural progenitor origin rather than derived from proliferating ESCs. Excessive growth of grafts and proliferation of nestin-positive cells have also been observed in two recent studies [19,26]. Thus, not only undifferentiated hESCs but also proliferating neural progenitors can generate tumors.

To effectively reduce the risk of tumors following grafting of hESC-derived cells, a combination of strategies might be most fruitful. For example, extended *in vitro* differentiation can be employed first to reduce the number of undifferentiated hESCs. In a second step, drug-induced apoptosis of undifferentiated hESCs and FACS/MACS using positive or negative selection can be used to enrich the desired cell population and delete unwanted cells. Although such a protocol has yet to be developed, it could ultimately make ESC-derived cell transplantation safe for the recipient.

What is the impact of chromosome instability in hESCs on their utility in transplantation therapies?

Recent studies have begun to define the chromatin organization and regulatory mechanisms that distinguish stem cells from their more mature counterparts. In hESCs and neural stem cells, genes associated with differentiation and development are reversibly silenced to confer stemness [45]. If epigenetic integrity is compromised by mutations or other changes that affect epigenetic regulator function, stem cells will aberrantly proliferate, transform and display some hallmarks of cancer [46,47]. If the promoter DNA at these repressed genes is methylated, the cells could be locked into a stem cell phenotype and divide excessively following transplantation. Therefore, abnormal DNA methylation in hESCs might present a risk for tumor growth when grafting such cells or their progeny. Indeed, abnormal CpG island methylation is observed in long-term cultures of hESCs [48] and during neural differentiation of hESCs [49]. Several recent reports describe genetic changes in long-term cultures of hESCs [30,50–53] involving gain of material from chromosomes 12, 17 and X. These changes might inactivate mechanisms that normally put constraints on stem cell expansion or could even transform transiently amplifying cells (e.g. neural progenitors) so that they continue to proliferate instead of becoming postmitotic and undergoing differentiation. The tendency for hESCs to acquire such genetic changes during prolonged culture might limit their use in cell therapy. On the other hand, a genetic or epigenetic mutation that leads to a selective advantage for the undifferentiated stem cells does not necessarily affect the differentiated progeny. Therefore, the impact of different genetic and epigenetic changes in hESCs needs to be carefully studied in the future. In the context of neural transplantation, experiments are needed to address both the effects of such changes on the tendency for hESC-derived grafts to form tumors and on the detailed function of, for example, dopamine neurons derived from altered hESCs. Knowledge about the potential importance of epigenetic silencing might lead to more innovative approaches to remove aberrant progenitors from hESC cultures. Thus, understanding epigenetic differences between stem cells and

differentiated cells might help us to develop genetic tools to exert more control over ESC differentiation and lineage restriction/flexibility in self-renewing stem cells. Regarding stem cell banks, possible genetic and epigenetic changes of hESCs within cell stocks should always be carefully monitored. The ultimate goal would be to obtain hESC derivates that are well suited for cell therapy and safe to transplant.

Immune reaction, an issue after neural transplantation

The brain is an immune-privileged transplantation site. Nonetheless, strong immune responses still occur in the brain in neurodegenerative disorders [54] or after intracerebral neural transplantation [55,56]. Consequently, allogeneic hESC-derived cells and animal-derived products used during *in vitro* differentiation of the cells might trigger immune reactions and lead to graft rejection [57]. Therefore, feeder-free and xeno-free conditions need to be developed, and the alloresponses to the hESC-derived cells taken into account.

How can immune rejection of hESC-derived grafts be avoided? Immunosuppressive treatments are widely used to inhibit immune rejection resulting from the histoincompatibility of transplanted cells. Unfortunately, they do not fully prevent chronic rejection and they increase the risk of opportunistic infections [58,59]. Several approaches preventing rejection of hESC-derived grafts have been considered [60]. In theory, the availability of a bank of genotyped hESC lines that at least partially match the genotype of graft recipients could reduce immune rejection. The creation of such a bank of human leukocyte antigen (HLA)-typed hESCs might be feasible [61,62]. Another approach to reducing immunogenicity of graft cells is to remove major histocompatibility complex or immune response-related components. Taken to the extreme, hESCs that are perfectly genetically matched to the host can be generated by somatic cell nuclear transfer. Such cells would be identical to the recipient except for proteins encoded by the mitochondrial genome [8,63]. However, this approach is probably not suitable for all PD patients, because some of them carry a gene mutation in their somatic cells.

The immune system of the host might affect the likelihood of tumor growth from stem cell-derived grafts. For example, proliferation of undifferentiated hESCs might be sensitive to inflammatory cytokines. Indeed, proliferation and differentiation of neurons from neural stem cells (adult neurogenesis) is either promoted or inhibited by inflammation [64,65] as a result of the release of growth factors such as BDNF, TGFβ and IGF-1 [66]. Changes in inflammatory status of the brain owing to the underlying neurodegenerative disorder or graft surgery could affect the local hospitality to the graft. In conclusion, a deeper understanding of the immune response in intracerebral transplantation is important to facilitate development of methods that improve graft survival and safety.

Turning human embryonic stem cells into a cell therapy: conclusion and future perspectives

We have highlighted the major challenges that currently face the use of hESC-derived cells in neurological diseases such as Parkinson's disease. The first challenge is to generate a large number of a defined cell population under feeder-free and xeno-free conditions. The second is to enhance cell survival after transplantation by optimizing differentiation *in vitro* and generating stable cells. The third is to develop protocols that eliminate unwanted cells, such as undifferentiated hESCs and rapidly proliferating neural precursors. Fourth, it is necessary to monitor genetic and epigenetic alterations in hESC-derived cells to ensure that they are safe to transplant. Finally, an important challenge is to prevent immune rejection of grafted hESC-derived cells.

Further research is needed to define whether transplanted hESC-derived neurons can integrate with host brain tissue, function and survive long term. Some argue that hESC-derived neural precursors survive transplantation better than postmitotic neurons; however, more evidence is needed to support this claim. The key factors and molecules that support hESC proliferation and differentiation

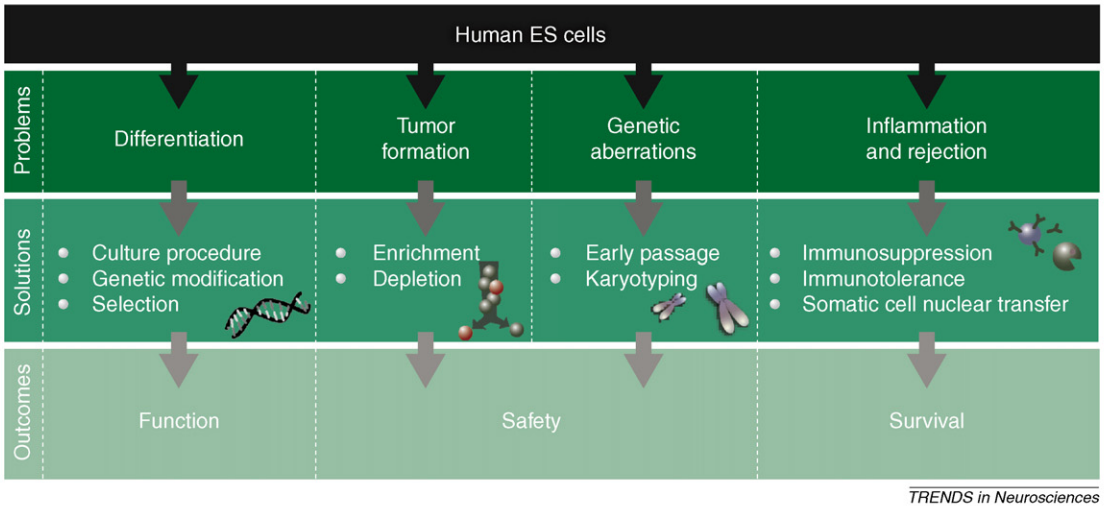

Figure 2. Steps required for safe and functional transplantation of hESC-derived cells. Transplantation of hESCs in the CNS is a promising therapeutic approach. However, there are still many challenges to overcome. These problems as well as their respective solutions are summarized here. As ultimate goals, these strategies will improve survival and functionality of grafts and the safety of the transplantation warranting the long-lasting effects of this therapeutic approach (for details, see text).

into neurons must be identified to generate cells suitable for transplantation. To create safe cell therapies suitable for neurological conditions, it is also necessary to eradicate risks such as tumors, karyotypic abnormalities and vigorous immune responses (Figure 2). To achieve these goals, basic and clinical translational research scientists need to interact. Patients suffering from neurological conditions such as PD, stroke or spinal cord injury might one day reap the benefits from such efforts.

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