CHAPTER

7

Strategies for bringing stem cell-derived dopamine neurons to the clinic: A European approach (STEM-PD)

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Abstract

The treatment of Parkinson's disease (PD) has for over 50 years relied on dopaminergic therapies that are highly effective especially in the early years of the condition, but ultimately are limited by the development of side effects that relate to the nonphysiological stimulation of dopamine receptors including in nonstriatal areas. Targeted regenerative therapies designed to restore specifically the lost dopaminergic innervation of the striatum would therefore represent a major advance in treating PD. Transplantation of human fetal ventral midbrain tissue to the striatum of PD patients has provided proof-of-principle that such an approach can provide long-term clinical benefits with a reduced dependency on any oral dopaminergic agents. However, fetal tissue is associated with several ethical and logistical problems and therefore does not represent a realistic route to the clinical treatment of PD in the future. As a result, alternative cell sources have been explored and the methods for producing authentic midbrain dopaminergic neurons from pluripotent cells have now advanced to a stage which makes it possible to efficiently and reproducibly produce DA progenitors at a much higher purity than can be obtained from human fetal tissue. A stem cell-based therapy for PD therefore has the potential to circumvent many of the problems currently associated with fetal tissue grafting. Here, we describe the challenges faced and the strategies that have been pursued in our European effort to bring a human embryonic stem cell (hESC)-derived dopamine cell product to clinical trial for PD.

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Keywords

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1 PARKINSON'S DISEASE AND ITS CURRENT TREATMENTS

Parkinson's disease (PD) is a common neurodegenerative disease affecting approximately 1% of people over 65 years of age, and as such it is likely to become more common as the population ages and life expectancy increases (Foltynie et al., 2004). The pursuit of more effective treatments for age-associated diseases such as PD represents a major goal for healthcare practice, as it may not only lead to clinical benefit for millions of patients, but also large-scale societal and economic advantages. PD is characterized clinically by the development of bradykinesia, rigidity, and a resting tremor, and these motor features are attributed mainly to the progressive degeneration of the dopaminergic output from the substantia nigra to the striatum (Connolly and Lang, 2014). The cause for this progressive loss of nigrostriatal dopaminergic neurons is not understood, and while genetic forms of PD are now recognized, most cases (90%) are still sporadic in nature (Gasser et al., 2011). The treatment of PD has for over 50 years relied on alleviating motor impairment by using drugs that act on this dysfunctional network either in the form of the dopamine precursor, L-dopa or dopaminergic receptor agonists. These treatments are highly effective in the first years of disease, but are ultimately limited by the development of severe side effects such as on–off motor fluctuations, L-dopa-induced dyskinesias (LIDs), hallucinations, and a range of behavioral and neuropsychiatric problems related to the nonphysiological stimulation of central dopamine receptors, including those found in nondopamine depleted areas of the brain (Connolly and Lang, 2014). In addition, patients continue to develop pathology at other CNS sites which leads to a large number of dopamine resistant nonmotor features including problems with mood as well as autonomic abnormalities. Nevertheless, it is well known that in the early stages of disease, especially in the younger patients, L-dopa can restore the patient's clinical state back to near normality.

Currently, as patients advance in their disease and enter a phase of the illness where motor fluctuations become more common, a range of more invasive treatments are considered. These include the use of subcutaneous apomorphine pumps, DuoDopa® (the delivery of a novel form of dopa directly into the small bowel) and deep brain stimulation of the subthalamic nucleus or internal part of the globus pallidus. While effective in the right patient, these therapies are expensive and not without side effects. In addition, these treatments still only target symptomatic elements of the condition and are not curative or disease modifying. As such, targeted dopamine replacement specifically at the site of the greatest dopaminergic denervation (i.e., the striatum), using a physiological delivery system, would

represent a major advance in the symptomatic treatment of PD. One way of doing this is by replacing the lost endogenous nigral DA neurons with transplants of putamenally placed replacement DA neurons, thereby generating permanent restoration of this critical element of the damaged brain circuitry seen in PD. Furthermore, if employed early in the disease course this approach could, in theory, obviate the need for any dopaminergic drugs, thereby preventing the development of long-term complications and side effects, and the need for expensive advanced therapies such as DuoDopa®, deep brain stimulation (DBS), and apomorphine. As such, a DA cell therapy has the potential to dramatically alter the natural history of the treatment of PD. This is especially the case for younger patients who have the prospect of living with the disease for several decades, and therefore represent the patient population who will benefit most from such an approach.

2 CELL REPLACEMENT STRATEGIES FOR PD

During the 1980s and 1990s, researchers at Lund University pioneered cell replacement therapy for PD using fetal ventral mesencephalic (VM) dopamine cells, which encouraged many other groups around the world to test different potential dopaminergic cell sources as a means of restoring dopamine levels back to normal in the parkinsonian striatum. Some of these strategies included the transplantation of peripheral catecholaminergic cells from adrenal medullary tissue and carotid body (Arjona et al., 2003; Goetz et al., 1989, 1991; Hurtig et al., 1989; Kelly et al., 1989; Kordower et al., 1991; Lindvall et al., 1987; Minguez-Castellanos et al., 2007) but also nonneuronal cells such as engineered retinal pigmentary epithelial cells (Spheramine®) and mesenchymal stem cell have been tested in clinical trials (Gross et al., 2011; Venkataramana et al., 2012). Of all these different strategies, it was really only those that employed fetal human VM tissue where long-term clinical improvement could be observed, consistent with the fact that this was also the only cell source which showed strong preclinical efficacy in animal models of PD (Barker et al., 2015).

In the late 1970s and 1980s, a number of landmark studies led by the team in Lund, and confirmed in many other labs, showed that allografted fetal VM tissue could:

- **(i)** survive in the adult rodent brain long term and differentiate into dopaminergic neurons with fiber outgrowth into the host striatum (Bjorklund et al., 1980);
- **(ii)** form synapses with, and receive synapses from, the host brain (Bolam et al., 1987; Freund et al., 1985);
- **(iii)** release dopamine in an appropriately regulated manner (Strecker et al., 1987); and
- **(iv)** restore function in animal models of PD that mimics the lost nigrostriatal pathway (Dunnett et al., 1983).

These capacities were dependent on the age of the tissue grafted, as younger and more proliferative donor tissue would give rise to better graft survival and fiber outgrowth (Bye et al., 2012; Seiger and Olson, 1977; Torres et al., 2007, 2008), as well as the site where the tissue was grafted, as grafts in different regions of the striatum would affect different functional deficits (Mandel et al., 1990). Furthermore, the speed with which the grafts could restore function in the lesioned rodent was also dependent on the species being used to provide the donor tissue, as tissue derived from larger, more slowly developing species—in particular human tissue—would require significantly longer in vivo maturation time to exert full functional effect (Bjorklund and Isacson, 2002; Dunnett and Bjorklund, 1999).

This ability for allografted fetal VM tissue to provide functional recovery was also shown in many other labs as well as in other species such as marmosets (Annett et al., 1995)—all of which gave confidence that this approach could work as a dopaminergic therapy in PD patients. Thus the early clinical programs using this approach launched in the mid- to late-1980s were built on a robust, reproducible preclinical dataset.

The cells used in these early clinical trials were obtained from subdissected VM tissue obtained from aborted human fetuses. Apart from the ethical and societal concerns associated with such cells, the clinical trials also revealed that this approach generated inconsistent results. While some grafted patients responded extremely well for many years allowing them to come off their dopaminergic medication (Kefalopoulou et al., 2014), with evidence of excellent long-term graft survival on scanning and at postmortem (Hallett et al., 2014; Li et al., 2016), other patients had no or only modest clinical improvements with significant side effects in the form of graft-induced dyskinesias (GIDs) (Freed et al., 2001; Ma et al., 2010; Olanow et al., 2003). This has led some commentators to conclude that this therapy is essentially not viable given the high variability observed in clinical efficacy and graft survival as well as the significant side effects seen in some patients (Olanow et al., 2009). Additionally, PD patients are known to have nonnigral dopaminergic pathology from disease onset with associated symptoms which will obviously not be targeted by this cell therapy approach (Kordower and Olanow, 2016; Olanow et al., 2009). Although these observations are valid, alternative explanations exist for the discordant results and poor graft survival and functionality seen in some patients which relate more to procedural issues such as:

- aspects of tissue preparation including the amount of tissue grafted and its storage prior to transplantation—factors which can compromise the number and health of grafted dopamine cells;
- the route by which the tissue was placed across the striatal target and how it was distributed to ensure even innervation of the target structure;
- the immunotherapy used to support the graft postimplantation (with none or very short courses of Ciclosporin only, in two of the biggest trials)—which can affect its protection from immune rejection and thus the number of surviving functional dopaminergic neurons (Freed et al., 2001; Olanow et al., 2003);

- the use of patients with extensive dopamine loss outside the dorsal striatum, which would limit the impact of any graft placed only in the posterior putamen (Piccini et al., 2005);
- the primary end point used in the trials in terms of the clinical measures chosen and the timepoint of assessment relative to graft implantation. These aspects of trial design are critically important, given (a) that some end points are more subjective in nature and (b) that the grafted dopamine cells can take several years to mature and fully integrate into the host striatum (Piccini et al., 1999); and
- the overall trial design in terms of the number of patients grafted and the number of trial arms which can affect the power of the study to detect any statistically significant effects.

Thus there are many reasons why these later transplantation trials could have failed to produce the results seen with the open label studies, although more recently another possible explanation has been suggested. In 2008, it was discovered that the grafts can acquire alpha synuclein pathology, which has raised the possibility that this might limit their efficacy, especially in the long term. While there is now convincing evidence that human fetal grafts do indeed adopt the alpha synuclein pathology of the host brain (e.g., Kordower et al., 2008; Li et al., 2008, 2016), it is also clear that the total number of cells in the transplant affected by Lewy Body pathology is small and that many patients in spite of this derive long-term benefit from their transplant (Kefalopoulou et al., 2014). In fact, recently the brain from a patient grafted 24 years ago came to postmortem analysis. The patient had shown a dramatic and long-term clinical benefit and increased DA transmission after transplantation (Piccini et al., 1999), and at postmortem had a large surviving graft with healthy DA neurons, of which the vast majority did not display any pathology (Li et al., 2016). Thus, while this latter aspect of graft pathology is of great interest in better understanding the pathogenesis of PD and in generating new studies and ideas around the prion-like behavior of alpha synuclein (Guo and Lee, 2014), these findings do not fundamentally undermine the strategy that began over 25 years ago with VM tissue transplants, given the sustained clinical benefits and long-term graft survival that have been reported from many of the trials over the years.

2.1 GRAFT-INDUCED DYSKINESIAS

In addition to the heterogeneity of clinical responses, there have also been concerns over the safety of fetal VM transplantation, given that a subset of transplanted patients developed off-medication-state GIDs (Freed et al., 2001; Hagell et al., 2002; Olanow et al., 2003). In a few cases, these adverse effects have been so severe that the patients have required additional neurosurgical intervention (Richardson et al., 2011). The reasons behind this emergence of GIDs are still debated but may relate to the tissue composition of the graft and its uneven placement, and thus

uneven pattern of innervation, across the striatal complex (Lane and Winkler, 2012). The GIDs were particularly severe in the Freed et al. study which transplanted "noodles" of VM tissue along the anteroposterior axis of the striatum using a novel transfrontal approach—an approach that was adopted following a significant hemorrhage in one of their open label patients grafted using the more standard neurosurgical approach (Freed and LeVay, 2002). This approach of Freed et al. clearly led to hot spots of dopamine innervation in the grafted striatum, and thus a theory arose that GIDs could be driven by overproduction of dopamine within localized areas of the striatum, thereby causing dyskinesias in the body parts mapped onto that striatal area (Ma et al., 2002). However, GIDs also appeared in a second NIH-funded trial, where a different transplantation approach was used (Olanow et al., 2003) which gave a more even dopaminergic innervation. In this trial, it has been hypothesized that the GIDs may relate to the uncontrolled release of dopamine from contaminating 5HT neurons within the transplant—neurons that take their embryological origin immediately adjacent to the midbrain dopamine cells. Thus it was proposed that the critical factor underlying GIDs was the ratio of 5HT to dopamine cells with high 5HT to DA cell ratios in the graft driving GIDs while high DA to 5HT ratios drive efficacy and no GIDs are seen (Barker and Kuan, 2010; Mendez et al., 2005; Politis et al., 2010). Importantly, preclinical animal studies had not previously revealed the emergence of GIDs, and this was therefore a unique side effect observed only in the clinical trials.

Although there is still some discussion as to the basis of GIDs, the information discussed earlier has led to an emerging concept that minimizing the presence of the 5HT neurons in the grafted VM tissue, while maximizing the number and optimizing the striatal distribution of dopamine cells is critical in going forward with new cell therapy trials for PD.

2.2 THE TRANSEURO FETAL VM TRIAL: A STEPPING STONE TOWARD THE NEXT GENERATION OF STEM CELL-BASED TRIALS FOR PD

To better understand and solve the issues of variability and adverse effects with fetal VM transplants in PD, an EU-funded multicenter study called TRANSEURO has been initiated (www.transeuro.org.uk, Figs. 1 and 4). This trial seeks to undertake a new and improved clinical trial design which will pave the way for more standardized and reproducible cell transplantation protocols for PD that can then be used for the next generation of stem cell-based therapies that are currently under development. This trial has sought to optimize patient selection, tissue preparation, tissue implantation, and support postgrafting as well as trial design in the following ways.

2.2.1 Patient selection

The optimal PD patients for any cellular reparative approach seem to be those that are younger with less advanced disease, and who are free of major LIDs (Barker et al., 2013). Thus, for TRANSEURO we have selected patients that are <65 years old,

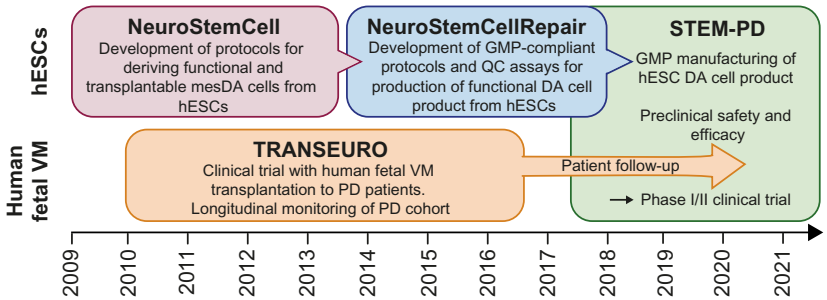

FIG. 1

Overview schematic of European preclinical and clinical programs leading up to a STEM-PD trial. Schematic shows how the previous and current FP7 EU-funded consortia NeuroStemCell, NeuroStemCellRepair, and TRANSEURO all contribute with important developments feeding into the STEM-PD program. *GMP*, good manufacturing practice; *mesDA cells*, mesencephalic dopamine cells; *QC*, quality control.

with disease duration of <10 years and no significant LIDs. They have to be free of any major cognitive or psychiatric problems. We have also selected patients that have the lowest risk of developing an early dementia based on some of our earlier epidemiological work using incident community-based cohorts of PD patients (Williams-Gray et al., 2007, 2009). Furthermore, based on previous PET studies that have shown that patients with extensive striatal dopamine loss do less well (Piccini et al., 2005), we have also only included patients with dopamine loss restricted to the dorsal striatum.

2.2.2 Tissue preparation

The fetal tissue needs to be grafted in a manner that ensures the optimal DA to 5HT ratio with sufficient dopamine cells grafted to achieve a clinical benefit. This is estimated to be around 100,000 surviving dopamine cells per hemisphere. We are thus using fetal VM tissue from at least three fetuses per side of the grafted PD brain with a dissection that is standardized and uses landmarks which seeks to minimize the number of 5HT neurons from the hindbrain. The tissue is stored for the minimal number of days necessary to enable this to be logistically feasible but without compromising the viability of the cells. This has involved using tissue collected from both medical and surgical terminations of pregnancy (Kelly et al., 2011) that is then stored in Hib E® at 4°C for no longer than 4 days prior to grafting.

2.2.3 Tissue implantation

The TRANSEURO trial uses a slightly modified version of the original Rehncrona instrument which has been shown to give good cell survival, distribution of dopamine neurons, and innervation of the target structure.

2.2.4 Immunosuppression

Tissue support in the form of immunotherapy involves using a slightly old fashioned triple regime (Ciclosporin; azathioprine and steroids) that previously has been adopted without major side effects, and evidence of graft survival without immune rejection (Barker and Widner, 2004). The immunosuppression is necessary for only relatively short periods of time as the BBB seals postoperatively as the grafts become integrated into the host brain. Thus we are using this regime for 12 months following the second graft, after which the therapy is slowly weaned off.

2.2.5 Trial design

In order to circumvent issues of sham surgery and placebo effects including investigator bias (Galpern et al., 2012), we have adopted a trial design where a large number of patients are followed in an independent observational study with assessments every 6 months. The patients are video recorded at all their assessments so that a third party can rate their motor examination, with the patient wearing a cap in all cases so that the third-party rater is blinded to their therapeutic intervention. Out of the initially enrolled cohort of 137 patients, a number ($n = 40$) have been selected for PET imaging of which roughly half have been randomized for grafting. The aim is to then compare the grafted patients against matched controls without the need for sham surgery (Fig. 4). The patients will then be followed up for 3 years and the primary end point will be their defined off UPDRS part III motor score at this time—a time at which it has previously been shown that the grafts are having a near maximal benefit to the patients (Piccini et al., 1999).

Despite the importance of this study in reestablishing the utility of cell-based therapies for PD, the fetal cell source is nonetheless still associated with ethical and societal issues, and fetal tissue transplantations are severely hampered by logistical problems associated with low tissue availability. Additionally, each patient will receive a unique cell suspension that is impossible to standardize given the tissue source. Thus, while TRANSEURO will be highly informative in understanding the response of different types of PD patients to transplantation and in optimizing the transplantation procedure, the fetal tissue source does not represent a realistic therapeutic option for the large numbers of patients with PD. To move to large-scale clinical applications, readily available, renewable, and bankable cells are needed in order to make this approach for PD a viable therapeutic strategy.

2.3 EFFORTS TO EXPAND HUMAN FETAL VM TISSUE FOR TRANSPLANTATION

As it became possible to expand neural stem and progenitors cells under growth factor stimulation without the need to first immortalize them (Pollard et al., 2006; Reynolds and Weiss, 1992), an obvious strategy to generate enough DA neurons for clinical use would be to expand the subdissected fetal VM progenitors in vitro. However, while some studies have succeeded in finding strategies that allow for short-term expansion of such fetal VM DA neurons (Ribeiro et al., 2013;

Studer et al., 1998), it has proven to be difficult to expand neural progenitors long term while maintaining their capacity to differentiate into authentic and functional DA neurons (Ribeiro et al., 2013, and references therein). Thus while the short-term expansion of fetal VM DA neurons is promising and would reduce the number of fetuses needed for transplantation, the manipulation of the cells during culturing means that the cell product would be classified as an advanced therapeutic medicinal product (ATMP). Therefore, the regulatory requirements for manufacturing and preclinical validation of each cell batch would be excessive in relation to the low number of cells derived with this approach, and these short-term expanded cells are thus not a feasible option for clinical use.

3 PROOF-OF-CONCEPT STUDIES FOR hESC-BASED DA CELL THERAPIES IN ANIMAL MODELS OF PD

Human embryonic stem cells (hESCs) have so far shown great potential in preclinical studies as a source of therapeutic cells to be used in cell replacement strategies for a number of diseases, thereby providing a good opportunity for scalable and traceable source of human cells (Trounson and DeWitt, 2016).

Following the initial success with hESC-derived differentiation and transplantation of neural cells to animal models of disease (Zhang et al., 2001), great hope was put into the use of these cells for treating neurodegenerative diseases such as PD. However, only more than a decade later, following a much better understanding of the normal developmental path for DA neurons in vivo (Arenas et al., 2015), did it become possible to generate transplantable dopamine progenitors of an authentic VM fate from hESCs and hiPSCs (Chen et al., 2016; Doi et al., 2014; Kirkeby et al., 2012; Kriks et al., 2011). In contrast to previous protocols, these differentiation protocols relied on patterning the cells toward a midbrain floor plate progenitor fate prior to transplantation, thereby ensuring an authentic midbrain DA phenotype of the mature grafted cells (Doi et al., 2014; Kirkeby et al., 2012; Kriks et al., 2011). When transplanting cells from these floor plate-based protocols to an in vivo model of PD, the 6-hydroxydopamine (6-OHDA) lesioned rat, the hPSC-derived DA neurons survived transplantation, innervated the host rat brain, integrated into the host circuitry, and provided behavioral recovery (Chen et al., 2016; Doi et al., 2014; Grealish et al., 2014, 2015; Kriks et al., 2011).

Given that the human fetal tissue is the only transplanted cell type which has so far shown long-term survival and efficacy in PD patients, we find it crucial to show that all new stem cell-derived DA products are tested in comparison with human fetal VM transplants and regard this as the gold standard for preclinical assessments. This comparison should include assessment of the key parameters for predicting functional efficacy in patients: (i) the ability of the grafted cells to completely reverse amphetamine-induced rotations in the 6-OHDA unilateral medial forebrain bundle lesion model of PD in cell numbers comparable to fetal cells (less than 1000 for full effect, Rath et al., 2013) and (ii) the ability of the cells to innervate

the host brain over sufficiently long distances needed to provide good innervation of the whole putamen in humans.

Indeed, parallel in vivo assessments of human fetal and hESC-derived DA neurons have shown that the hESC-derived DA neurons can function with equal potency to primary human fetal VM after transplantation in a rat model of PD (Grealish et al., 2014), and that they also have a similar innervation capacity and express identical markers of mature A9 and A10 neurons (Grealish et al., 2014; Kirkeby et al., 2012). In this regard, it is important to recognize that terminal maturation of the DA neurons takes place over many months and even years after transplantation. Although this is challenging to assess in xenograft models, it is absolutely necessary to perform long-term xenograft work in the preclinical evaluation of any such product, since terminal maturation in vitro cannot substitute for assessment of in vivo graft outcome, especially given that the expression of commonly used markers of mature DA neurons in vitro does not correlate with the DA yield of the transplanted cells in vivo (Kirkeby et al., 2017).

Based on our preclinical in vivo assessments (Grealish et al., 2014, 2015; Kirkeby et al., 2012, 2017), we find that the protocols for generating authentic and functional mesencephalic dopamine (mesDA) neurons from hESCs have now reached a stage of sufficiently high quality for moving forward toward a clinical trial. However, in order for such a trial to become a reality, it is first necessary to produce cells suitable for use in patients which includes good manufacturing practice (GMP) adaptation, GMP production, quality control (QC) development, and good laboratory practice (GLP)-standard preclinical safety and efficacy profile of the DA cell product. Below, we will outline the challenges we have met and the programs and strategies we have employed in order to bring our cells toward a first-in-human clinical trial in PD (the STEM-PD trial) in Europe (Fig. 1).

4 PRODUCING A CELL PRODUCT FOR THE STEM-PD CLINICAL TRIAL

4.1 CHOOSING THE RIGHT STEM CELL LINE

An important decision when producing a clinical stem cell product is choosing an appropriate stem cell line as starting material. This task may sound simple, but has in fact revealed itself to be exceedingly complex, in part because the regulatory requirements for donor eligibility and source testing have not kept up with scientific developments in the field (Couture and Carpenter, 2015). One early decision that has to be made is to determine the pluripotent cell source to be used: hiPSCs or hESCs. Whereas hESC lines are considered the gold standard for pluripotent cells, hiPSCs may circumvent many of the major ethical and political problems associated with hESCs, and may eventually even allow for patient-specific therapies with minimal need of immunosuppression. On the other hand, hiPSCs bring with them an increased risk of genetic and epigenetic abnormalities, and the long-term consequences of such potential abnormalities in grafted cells are still unknown. This issue therefore results

in extreme caution from a regulatory perspective, and contributed to the premature halting of the first-in-human hiPSC trial in Japan (Ipscell.Com, 2015). In addition, hiPSC strategies are associated with significantly increased costs due to the extensive safety and efficacy testing required on several different clonal cell lines prior to any clinical trial. Since there is clinical evidence that allogeneic neural transplants can survive in the brains of PD patients for >20 years with only short periods (6–18 months) of immunosuppression postgrafting (Li et al., 2016), we have for the STEM-PD trial chosen to use hESCs rather than hiPSCs as the clinical cell source in order to avoid the unknown risks and increased costs associated with hiPSCs.

Having made this decision, the next issue is to choose the most appropriate hESC line for clinical transplantation, and in this respect several aspects must be considered: (1) Mode of derivation: Is the line fully GMP-derived or is it research-derived and then GMP adapted? Since the regulatory requirements for GMP-grade cell derivation are different in the EU and the United States with regards to entering clinical trials and marketing a stem cell product, the issue of GMP-grade derivation may become important even after the cells have passed through the first phases of clinical trials. (2) Donor origin: Where are the gametes sourced and what is the geographic origin of the donors? Is the line derived from donors that comply with national regulations and international use? For example, gametes from a country which is regarded as BSE affected by the FDA (2007) means that these cells cannot currently be used in a phase I study in the United States. (3) Does the informed consent of the donors allow for use of the cell line in clinical trials and for marketing as a stem cell product? For many hESC lines, the informed consent is not clear on these issues, and this poses a risk that such lines will fail approval to enter the market—even after successful completion of clinical trials. (4) Is the cell line free of oncogenic mutations? Although this may seem to be a straight-forward question, choosing an appropriate screening for oncogenic mutations is not trivial. The most rigorous analyses involve whole genome sequencing; however, such analyses are bound to reveal SNPs and mutations which are difficult to interpret. Although such identified mutations may simply be a result of normal individual variation rather than of pathogenic events, the uncertainty associated with such results could eventually stop or halt the cells from being used in clinical trials (Ipscell.Com, 2015). In contrast, commercially available oncogenic single-nucleotide polymorphism (SNP) platforms only screen for known mutations, but are easier to interpret, and may therefore present a more favorable option. (5) Does the cell line generate mesDA cells at a high efficiency and purity? Naturally, a cell line which fulfills all of the above-mentioned criteria, but which cannot generate high-purity mesDA cultures, is not suitable for a clinical trial for PD. Thus, several cell lines need to tested in vitro and in vivo prior to choosing the line which has the best profile for a PD clinical trial.

In our EU-funded consortium NeuroStemCellRepair (Fig. 1), we have tested mesDA differentiation from several GMP-derived (or GMP-compatible) lines of UK and Swedish origin (from Sheffield, Roslin Cells, and Karolinska Institute), and we have shown that in particular the RC17 line from Roslin cells and the GMP-compatible HS980 line from Karolinska Institute are promising with regards

to mesDA purity and in vivo efficacy (Kirkeby et al., 2017 and unpublished data). We have further tested the RC17 line extensively to confirm batch-to-batch reproducibility of the GMP differentiation protocol (Kirkeby et al., 2017). Nonetheless, all of these lines are derived from donors within European countries which are listed as BSE affected by the FDA, and although they adhere to the highest standards of GMP-grade derivation, they are therefore likely to face challenges in being developed as a cell therapy for use in patients in countries outside of the EU.

4.2 PRODUCING A CLINICAL CELL PRODUCT

Developing efficient differentiation protocols for derivation of enriched cultures of DA progenitors from a GMP cell line is a first step toward producing a clinical cell product. However, prior to full GMP production, the entire cell culturing and differentiation procedure must be adapted to a GMP standard, and then transferred to a GMP production facility (Fig. 2). This may prove to be a tedious and time consuming process, since the replacement of reagents and suppliers for crucial elements of the protocol can result in altered differentiation outcomes, and subsequently the whole process may need to be optimized again based on each and every newly replaced reagent. This is particularly true for VM differentiation protocols, since even small differences in the potency of each patterning reagent may shift the fate of the cells toward more rostral, caudal, or lateral neural fates (Kirkeby et al., 2012). To adapt our VM differentiation protocol to GMP standards,

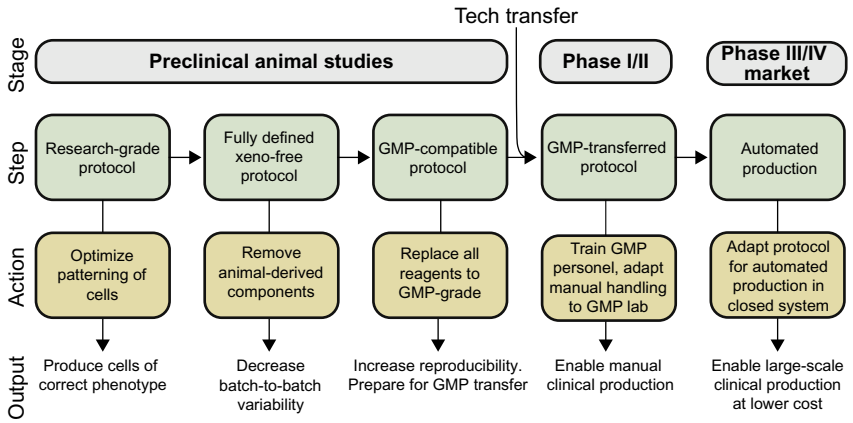

FIG. 2

Schematic outlining the process development steps for manufacturing of a clinical cell product. The figure shows the process development steps that are necessary to pass through in order to enable clinical production of a stem cell product when starting from a research-grade differentiation protocol.

we have implemented the following changes from our original research differentiation protocol (Kirkeby et al., 2012):

(1) switching the pluripotency maintenance conditions to culturing on Lam-521 in StemMACS^{TM} iPS-Brew XF, as these reagents are already produced or under development as GMP products (MX-521 and iPS-Brew GMP);
(2) eliminating the embryoid body step in order to reduce batch-to-batch variation, with a shift in the protocol to entirely adherent differentiation;
(3) replacing the animal-derived laminin coating in the differentiation protocol to recombinantly produced Lam-111 coating;
(4) adding a ROCK inhibitor to the cells at the days of passaging to reduce batch-to-batch variation in cell yield at the time of replating; and
(5) replacing research-grade reagents to new reagents at GMP grade or Thermo Scientific CTS grade (see reagents in Kirkeby et al., 2017).

Implementation of these changes to the protocol has significantly increased reproducibility and also the yield of differentiated progenitors compared to our research-based protocol, and we can now produce on average >300 million progenitors on day 16 of differentiation (day of transplantation) when starting with just 1 million undifferentiated progenitors (Kirkeby et al., 2017 and Fig. 3). In addition, a cryopreservation procedure of the final cell product has now been developed and tested repeatedly to ensure maintenance of efficacy and safety in the cryopreserved product. We have

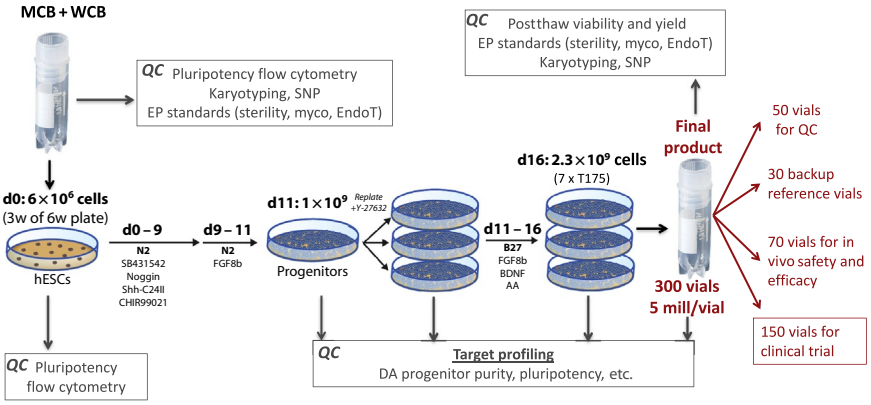

FIG. 3

Overview of process development steps for manufacturing of a clinical cell product. Schematic shows overview of the GMP protocol scaled for production of the final clinical batch, including indication of critical QC steps in the process. The final GMP protocol has been assessed to produce a very high yield of DA progenitors, which enables manual production of the full clinical batch in only 7 × T175 flasks at peak production (Kirkeby et al., 2016). *EP*, European Pharmacopoeia; *MCB*, master cell bank; *SNP*, single-nucleotide polymorphism; *WCB*, working cell bank.

also now validated the in vivo functionality of the cryopreserved cells upon transplantation by comparing cryopreserved and fresh cell grafts. This is critical to ensure that the cells can be banked and then easily distributed for direct clinical delivery at multiple clinical centers without the need for further culturing or manipulation of them at these clinical sites.

For the STEM-PD clinical trial, we will produce one large batch of a DA cell product of 300 cryopreserved vials with 5 mill cells per vial. Given the highly efficient yield and small culture volumes to achieve this, it is possible at this stage to use a manual system in a GMP facility without the need for developing complex automated procedures adopting closed systems of production. The clinical batch is projected to be sufficient to allow for all the in vitro QC assays, sterility testing, preclinical safety, and efficacy testing needed to file for a clinical trial as well as provide enough cells to conduct phase I/II clinical trials (Fig. 3). Using this setup will ensure that the exact same cells that will be transplanted to the first PD patients have first been extensively tested and validated in in vivo animal models, and by doing so, this minimizes the risk of subjecting patients to any unpredictable risks associated with batch-to-batch variations in the differentiation procedure.

4.3 DEFINING THE CLINICAL PRODUCT COMPOSITION

A major concern of using a pluripotent cell source for transplantation is the possibility that the graft will be contaminated with remaining pluripotent cells and/or aberrantly differentiated cells of nonneural origin. Naturally, screening for the most obvious sources of contaminating cells—in particular pluripotent cells and early cells from different germ layers—can be included in the QC assessment of the product, but complete characterization of the cell composition has not been performed to date. Here, it is worth noting that despite the fact that human fetal tissue grafts are very heterogenous and only a minor component of the grafts are DA neurons (Thompson and Bjorklund, 2012), the lack of knowledge on the full cellular composition of fetal cell suspensions has not precluded these cells from being used in clinical trials. In comparison, the hESC-derived VM cultures contain a higher purity of midbrain floor plate cells than subdissected human fetal VM (20–40% in human fetal VM vs 80–90% in hESC-derived populations; Kirkeby et al., 2012, 2017), and it is therefore likely that correctly patterned hESCs will give rise to less heterogeneous grafts. On the other hand, the non-DA neuronal components of the grafts are likely to be different between fetal and ESC grafts, for example, fetal grafts contain a large glial component that is not seen in most ESC-derived grafts (Grealish et al., 2014), and fetal VM obviously runs no risk of containing pluripotent cells.

As in the case of fetal cell grafting, we do not expect complete characterization of each and every cell in the final product to be a requirement by the regulatory authorities for going into a clinical trial with an hESC-derived product. Nevertheless, safety is the main concern, and the absence of pluripotent and tumorigenic

cells will need to be specifically assessed through in vitro assays as well as extensive long-term in vivo tumorigenicity studies. For future clinical development, complete product characterization of the transplanted product and of the mature graft derived from it through for instance, single cell RNAseq will be useful in providing information on whether the product contains small populations of cells which may pose a risk if present at higher proportions—including 5HT neurons in the case of midbrain patterned derivatives from stem cell sources for use in patients with PD. Gaining knowledge on the exact identities and marker profile of such unwanted populations, will make it easier in the future to survey and analyze batches of the cell product during the production process. This way, batches with high levels of aberrant cells can be discarded prior to going through the expensive phases of product storage, QC, and in vivo testing.

For the STEM-PD trial, we will be performing QC and target validation of our cell product based primarily on flow cytometric assessments. Whereas bulk qRT–PCR-based readouts cannot discriminate between batches containing few cells with high gene expression and batches containing many cells with low gene expression, flow cytometry can provide an accurate quantification of the percentage of positive and negative cells for each target marker, and it thereby provides accurate information on the purity of the cell product. Using flow cytometry, we will analyze the purity of our DA cell product, prefreeze and postfreeze, and we will verify the absence of pluripotent cells by analysis of OCT3/4. Additionally, we will complement the flow-based QC assays with qRT–PCR analysis for the expression of markers which we have recently identified to correlate negatively and positively with in vivo graft outcome (Kirkeby et al., 2017). The final cryopreserved cell product will go through additional rounds of target profiling, sterility testing, and viability assessments prior to batch release.

4.4 PRECLINICAL SAFETY AND EFFICACY TESTING

Once a final GMP-produced cell product is ready and has passed through all the necessary in vitro QC release assays, it will proceed to preclinical safety and efficacy testing prior to being used in a clinical trial. The extent and design of such studies required will be planned in conjunction with the guidelines and recommendations of the relevant local authorities. Based on the initial interactions with such agencies, safety testing will be composed of standard in vitro testing, as well as tumorigenicity, biodistribution, and toxicology studies in intracerebrally transplanted male and female rats in a dose escalating design with 1, 3, and 6 months as the end points for analysis.

In addition to safety studies, it is also pertinent to show efficacy of the stem cell product in relevant animal models of PD. Our minimal criteria for proceeding with the cell product to clinical trial will include: (i) the ability of the cells to mediate complete functional recovery of amphetamine-induced rotations after intrastriatal grafting in the unilateral 6-OHDA depletion model in rats and (ii) the ability of the cells to innervate the host brain at a scale relevant for the human brain. Long-range

innervation cannot be assessed from standard striatal graft placement in the rat, but may instead be assessed through intranigral grafting paradigms (Grealish et al., 2014) or in large animal models such as primates or pigs.

The functional potency of the cells is another important factor which needs to be determined in the preclinical studies, as this will guide the dosing of the cells in the first-in-human studies. It has been established that research-grade hESC-derived DA neurons function at equipotency to human fetal cells (Grealish et al., 2014), and this has to be evaluated also for the GMP manufactured product. Postmortem brain analysis of PD patients transplanted with human fetal VM tissue, indicate that grafts containing approximately 50–100,000 tyrosine hydroxylase-positive (TH^+) neurons per hemisphere are enough to obtain good graft function and clinically meaningful effects (Hauser et al., 1999; Li et al., 2016; Mendez et al., 2005). The appropriate cell dose to use in patients will therefore be guided by the graft outcome parameters in the preclinical in vivo efficacy study, in particular the potency assay, with 100,000 TH^+ neurons per graft as a target dose. To this end, the dopaminergic yield of the clinical cell product (i.e., the number of TH^+ neurons obtained in grafts per 100,000 grafted cells) needs to be determined. From our current rounds of preclinical assessments, we estimate that the target dose for clinical trial will be around 2 million grafted cells per hemisphere (Kirkeby et al., 2017).

5 THE STEM-PD CLINICAL TRIAL DESIGN

5.1 CLINICAL TRANSPLANTATION PROCEDURE

The delivery of the stem cell-derived product to the brain—in terms of how the cells will be implanted and where—is likely to be no different from the strategies used in the successful open label trials with fetal VM tissue and our ongoing TRANSEURO trial. The key issues will be ensuring that the cells are delivered: (i) in the right numbers and (ii) evenly across the target structure to ensure near complete homogeneous innervation of the putamen. The stem cells will therefore most likely be delivered using the modified Rehncrona instrument employed in our ongoing TRANSEURO study. The cells will be delivered along six tracts with small deposits made at multiple sites along each tract, to ensure that the cells are evenly grafted and spaced such that their fiber outgrowth will innervate the striatal area between these tracts. This proposed protocol is predicated on the grounds that the GMP manufactured dopamine cell product will behave like fetal VM dopamine cells in terms of axonal outgrowth, which is something which needs to be validated in the preclinical efficacy tests.

5.2 MEASURES TO AVOID THE DEVELOPMENT OF GIDs

One of the major issues with the fetal VM trials relates to the development of GIDs postgrafting. This has been thought to relate to the unequal innervation of the parkinsonian striatum by the transplant as well as the presence of contaminating 5HT

neurons in the graft (see Section 2.1). In the case of a stem cell-derived dopamine transplant, the issue of contaminating 5HT neurons can be avoided as the optimized differentiation protocols ensures contaminating hindbrain cells will not be generated (Kirkeby et al., 2017). In addition, to achieve good graft function and to avoid potential dopamine hot spots in the grafts, it is important to ensure that the hESC-derived DA neurons can innervate long distance and not just around the site of implantation. Initial assessments of this in animal models confirm that the implanted hESCs have the potential to innervate >10 mm from the graft site when grafted at the progenitor stage (Grealish et al., 2014). Based on our preclinical assessment (Grealish et al., 2014; Kirkeby et al., 2017), we therefore expect that the grafted hESC-derived DA neurons can provide sufficient and even innervation of the grafted putamen, although this will have to be confirmed in the first clinical trial.

Finally, it has been shown preclinically that GIDs are only generated in animals with already established LIDs (Garcia et al., 2011), although any emergence of GIDs in animal models may be different to that seen clinically as they are triggered only by stimulation with amphetamine. Nonetheless, this has led in the TRANSEURO study to the recruitment of patients with no significant LIDs. For a first-in-human study with a stem cell-derived dopamine cell it will most likely be required to test the treatment on more advanced patients, given the favorable therapeutic options for patients at earlier stages of the illness, and the experimental nature of the therapy being trialed. Thus it is likely that patients with LIDs will be recruited to our first trials, in much the same way as was done for patients in the early stages of human fetal VM grafting (Lindvall et al., 1990). However, this will be revisited and refined as we move forward with this treatment into phase II/III trials, especially as ultimately one imagines that this therapeutic agent could be used as a first line treatment for newly diagnosed PD patients.

5.3 CLINICAL TRIAL DESIGN

The design of a first-in-human trial is such that the tolerability (safety) and feasibility of grafting the cell product are the primary objectives rather than efficacy, which is more the domain of phase II/III studies. Thus the proposed phase I study will seek to enroll only a few patients ($n = 3 + 3$ design, see Fig. 4) which will have a dose finding element, as well as a large number of safety measures especially around the risk of abnormal cell proliferation at the site of grafting. However, it is important to remember that this will not be the first ES cell product to be trialed in patients, as such therapies have already been tried clinically before, albeit not in PD but spinal cord injury and retinal disease (Clinicaltrials.gov, 2010; Schwartz et al., 2015; Song et al., 2015).

5.3.1 Patient selection and dose

It is likely that the patients recruited to a first-in-human clinical trial for PD will be in the mid-stage of the disease given the high risks associated with any first-in-human trial. We see the patients already enrolled in the TRANSEURO

observational arm to be ideal for our first stem cells trial. This has the advantage that the patients will have been extensively followed pregrafting and well characterized and thus the trajectory of their disease course well defined. Secondly, we will, at some level, be able to compare our stem cell transplants against fetal VM grafted patients, although of course they will have been grafted at slightly different stages of disease.

Using this as our starting point, the patients will have PD as defined by the standard Queen Square Brain Bank (QSBB) criteria, and be less than 70 years of age with a disease duration in the region of 10–15 years. They will have shown a clear response to dopaminergic therapies and be free of major psychiatric and ongoing unstable medical disorders, as well as cognitive deficits predictive of an early PD dementia (semantic fluency and visuospatial deficits). Owing to their more advanced disease stage, they are likely to display some LIDs, and approaching the time at which more invasive therapies for controlling these are being considered. Thus, another benefit of this trial design will be that it allows for us to make some inference as to how our new stem cell therapy compares with already established interventions such as DBS and DuoDopa®, as well as the best medical therapy given that some of our other patients within the TRANSEURO observational cohort, not recruited to the stem cell trial, will be in receipt of these treatments (Fig. 4).

Other exclusion criteria will include structural imaging (MRI) to rule out other significant intracranial pathology as well using F-dopa PET to exclude patients with significant ventral striatal dopamine loss.

The number of patients that will be recruited is a moot point. It is envisaged that the first three patients will be grafted with the lowest dose of cells that we think will be efficacious, namely 1–2 million cells to reach a total of 50–100,000 mature TH^+ neurons in the grafts. If no adverse effects is seen at 12 months (which is also the timepoint where we expect to see the first indications of efficacy by PET), then a decision will be made as to whether to stay at this dose of cells or increase it for the next cohort of three patients. At this point, a consensus will be needed as to whether the final part of a phase I study should involve another cohort of say six patients grafted with this higher dose assuming the exploratory study was without major complication. This input will be provided by an independent external advisory board of experts once the trial has been initiated.

5.3.2 Clinical assessments

The TRANSEURO trial has resulted in a novel battery of clinical assessments which will also be suitable for a stem cell-based trial. In addition, a trial using a pluripotent-derived cell product will need some additional safety measures. Thus, all patients will be tested on a range of motor tasks including timed motor tests as well as the new Movement Disorder Society Unified Parkinson's Disease Rating Scale (UPDRS) in the on and defined off state (defined off being without dopaminergic medication from midnight on the day of the assessment which will done first thing that morning. Long acting dopamine drugs will be stopped for at least 24 h prior to

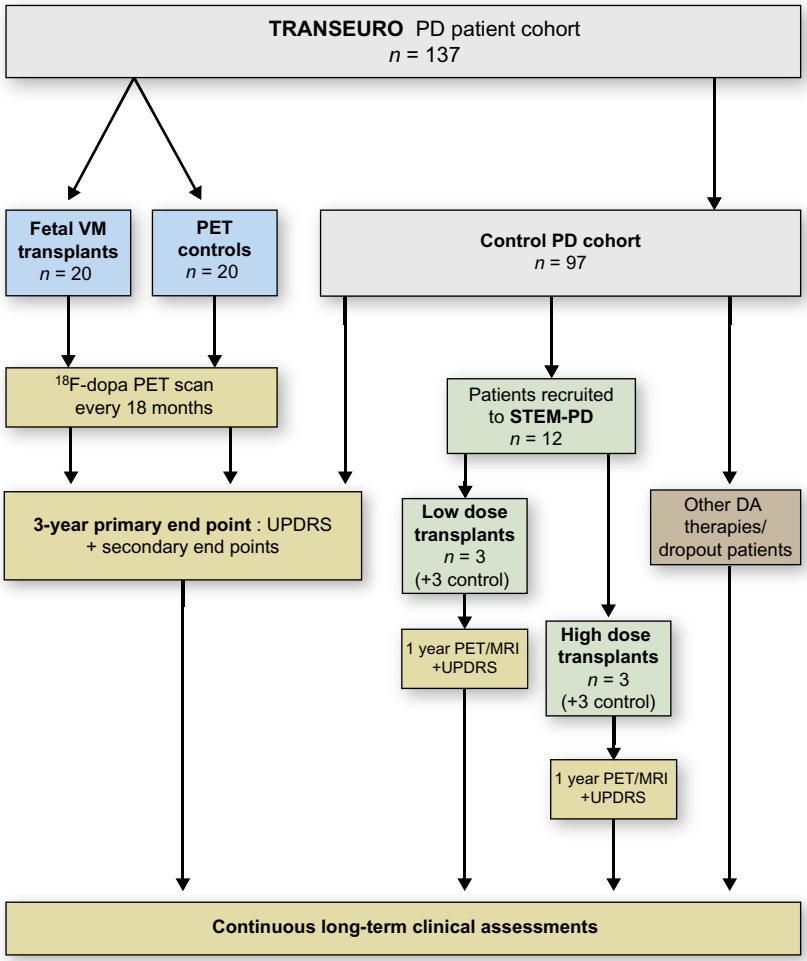

FIG. 4

Overview of patient flow from TRANSEURO to STEM-PD. Schematic shows how the patients for the STEM-PD trial will be drawn from the TRANSEURO PD patient cohort, as this is a patient cohort which has already been assessed longitudinally as part of the TRANSEURO observational study. The transplanted patients will be compared to the control cohort which will receive various different standard DA therapies, including DBS. *UPDRS*, Unified Parkinson's Disease Rating Scale.

this assessment). A range of cognitive and neuropsychiatric tests will also be administered including the revised Addenbrooke's Cognitive Examination, the Montreal cognitive assessment, and the NeuroPsychiatric Inventory as well as the Beck Depression Inventory.

Other assessments will include routine blood tests linked to the immunosuppression as well as imaging to look for graft overgrowth (using MRI) and dopaminergic cell survival and innervation using F-dopa PET and graft integration and graft placement using functional MRI.

They will have clinical assessments as detailed above at 6 and 12 months, with MRI at 3, 6, and 12 months and PET at 12 months, and will subsequently be assessed every 6 months for at least 3 years posttransplantation so that some comparison can be made with the TRANSEURO fetal VM grafted patients.

From past fetal tissue studies, the ability to study the cell survival and innervation has become extremely informative in designing TRANSEURO as well as future stem cell trials, and declaration of intent from all patients to collect their brains when they die for postmortem analysis will be pursued.

5.3.3 Immunosuppression

Prior to the first-in-human trial it is unknown whether the stem cell-derived dopamine cells have the same immunogenicity as fetal VM tissue as well as whether the immunotherapy itself could affect the differentiation of the stem cells. These questions are currently being investigated in experimental studies along with whether newer regimes of immunosuppression using tacrolimis and monoclonal antibody therapies targeted to T cells may be a better option than ciclosporin A, azathioprine, and steroids used in the fetal trials. If these studies should fail to identify better immunosuppression regimes for hESCs, we will implement the same immunosuppression regime as that adopted in TRANSEURO (see Section 2.2.4).

5.3.4 Trial end points

At this stage of development of a new therapy, the critical factor will be safety/tolerability and feasibility. Thus, the primary end points will be that there are no major adverse events either in terms of what is seen at the graft site on imaging and/or what is seen clinically with the patients. When looking to find the optimal dose of cells, the question arises as to how long it takes to achieve the maximal benefit from the graft. This is difficult as, with fetal VM tissue this can take as long 3 years, and possibly even longer (Kefalopoulou et al., 2014). However, it is also known that F-dopa imaging improves ahead of clinical improvement and thus it is likely that at 12 months one will get sufficient information to make an informed decision about whether one now needs to, or can move to, a higher dose of cells in the next patient cohort.

It will be particularly interesting to follow the clinical course of the first transplanted patients over at least the first 3 years postgrafting, given this is the time of the primary end point of the TRANSEURO transplant trial. Ideally, the patients should be followed for the rest of their lives as has been done in some of the open label studies using fetal VM tissue (Li et al., 2016).

6 CONCLUDING REMARKS: WHERE ARE WE NOW?

The field is at an exciting stage of development and several research consortia worldwide are developing stem cell-based therapies for PD that are expected to reach clinical trials within the next few years—and the patient community is carefully following these developments. Under such circumstances, it is important to move at the fastest possible speed while always keeping safety and quality in mind and ensuring the clinical development is supported by robust preclinical data. To balance this, our EU-based networks have initiated the establishment of a global consortium that brings together the major teams working on developing stem cell-derived neural transplantation therapy for PD—GForce-PD. GForce-PD involves multidisciplinary teams from Europe, the United States, and Japan, and together we work for better cells, better trials, and ultimately the best available therapy for individuals with PD (Abbott, 2014).

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