EXPERT REVIEWS

Cell replacement therapy for Parkinson's disease: how close are we to the clinic?

Expert Rev. Neurother. 11(9), 1325–1339 (2011)

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Cell replacement therapy (CRT) offers great promise as the future of regenerative medicine in Parkinson's disease (PD). Three decades of experiments have accumulated a wealth of knowledge regarding the replacement of dying neurons by new and healthy dopaminergic neurons transplanted into the brains of animal models and affected patients. The first clinical trials provided the proof of principle for CRT in PD. In these experiments, intrastriatal transplantation of human embryonic mesencephalic tissue reinnervated the striatum, restored dopamine levels and showed motor improvements. Sequential controlled studies highlighted several problems that should be addressed prior to the wide application of CRT for PD patients. Moreover, owing to ethical and practical problems, embryonic stem cells require replacement by better-suited stem cells. Several obstacles remain to be surpassed, including identifying the best source of stem cells for A9 dopaminergic neuron generation, eliminating the risk of tumor formation and the development of graft-induced dyskinesias, and standardizing dopaminergic cell production in order to enable clinical application. In this article, we present an update on CRT for PD, reviewing the research milestones, various stem cells used and tailored differentiation methods, and analyze the information gained from the clinical trials.

Keywords: cell therapy • dopaminergic neurons • embryonic stem cells • induced pluripotent stem cells • mesenchymal stem cells • neural stem cells • Parkinson's disease • regeneration • stem cells • transplantation

Parkinson's disease (PD) is a chronic progressive neurodegenerative disease that affects over 1% of the population over 65 years of age and has been positioned as the second most common neurodegenerative disorder after Alzheimer's disease [1]. The cardinal symptoms of PD include resting tremor, rigidity, bradykinesia and postural instability. Symptoms that appear later also include non-motor signs, such as autonomic, sensory, psychiatric and cognitive impairments [2]. The clinical motor dysfunction observed in PD is primarily the consequence of a progressive and selective degeneration of dopaminergic (DA) neurons in the substantia nigra pars compacta of the ventral midbrain, resulting in a severe deficiency of dopamine in the nigrostriatal pathway affecting the striatum [3]. Restoring dopamine levels by levodopa therapy is still the cornerstone of PD treatment; however, long-term treatment results in serious motor and psychiatric side effects, a process known as the 'L-DOPA paradox' [4]. Other treatments such as DA agonists, metabolic enzyme inhibitors and deep brain stimulation aid in management of PD patients; however, all these treatment options still have clinical limitations [5]. Despite the availability of effective symptomatic drugs, there is currently no cure for PD and almost every attempt to slow the neuronal loss or stop disease progression has failed [6].

Cell replacement therapy (CRT) has emerged as an attractive strategy for regenerative medicine in PD. As 'proof of principle', it was demonstrated in several clinical studies that have been performed that replacement of lost DA neurons can improve motor symptoms of PD patients. Although this approach has been investigated for over three decades, several obstacles have emerged and prevented its wide application in PD therapy, diminishing the initial excitement. New approaches are currently being developed and tested further in order to achieve a substantial improvement in CRT, enabling future use of these promising therapies for PD patients. The aim of this manuscript is to review the cutting edge of current research on CRT strategies in PD, as well as to describe the experience that has accumulated from clinical transplantation trials in PD patients.

 10.1586/ERN.11.74 ISSN 1473-7175

Cell replacement therapy & PD: milestones

Parkinson's disease is primarily characterized by a selective loss of a small population of a specific subset of neurons (A9 DA neurons), making it attractive to study the effect of direct replacement of new and healthy A9 DA neurons. Since the concept of CRT emerged as a successful therapeutic alternative in PD, many obstacles have restricted the direct translation to the clinic, making it more complex than was initially expected. The complexity of the experimental design in CRT, among others, still resides on which cells are best suited to provide functional A9 DA neurons and where to transplant them in order to re-establish the affected system.

In the mid-1970s, work carried out by several groups led by Olson, Seiger and Hoffer [7–10], as well as Björklund and Stenevi [11–13], initiated the concept of cell replacement in PD. The basic concept was to provide dopamine, through transplantation of dopamine secreting cells, in the caudate and putamen of the striatum. The striatal target was selected to ensure the prompt release of DA in the primary area affected by DA nigral cell loss. These works provided extensive evidence that grafted DA cells isolated from the ventral mesencephalon (VM) retained the capacity for long-term survival in the degenerated brain, reinnervate striatal targets and significantly improve motor functions [14]. Nigral transplantation was also tested, as this could be a more physiological approach. Although the cells survived in the nigra, they failed to properly extend axons through the nigrostriatal pathway and innervate the striatum, thus no functional recovery was documented [14–21]. As fetal tissue availability is limited and may present ethical implications, researchers searched for alternative sources of DA-secreting cells. Pioneering works, such as those made by Freed *et al.* [22], Backlund *et al.* [23] and Madrazo *et al.* [24], strengthened the potential of CRT for PD, using autologous grafts of the adrenal medullary tissue. Graft survival was observed in transplanted rats [22] and in MPTP-treated monkeys, and some symptomatic improvements but limited cell survival after transplantation, were demonstrated [25,26]. In 1987, the first clinical use of adrenal medullary graft was reported with significant improvements; however, later on, trials demonstrated less favorable outcomes [24,27]. Several longitudinal studies have analyzed over 300 PD patients grafted with tissue from different origins and showed that most of the patients with a positive clinical outcome during the off-drug period presented improved motor symptoms by 30–60%, as measured by the United Parkinson's Disease Rating Scale (UPDRS) [28]. Moreover, formed DA neurons from the transplanted tissue reinnervated the denervated striatum and became functionally integrated, restoring striatal dopamine release and giving rise to symptomatic relief. Implanted cells were detected 10 years after transplantation in the brain of treated patients [29]. However, two double-blind placebo-controlled studies using embryonic mesencephalic tissue transplantation in PD patients demonstrated disappointing results. There are several possible explanations for these results, but it clearly proved the need to develop other sources of DA neurons, and refine both the transplantation techniques and suitable patient selection. Owing to the limited availability of embryonic tissue, the possible ethical implications and the high variability of functional outcome after transplantation, future CRT in PD needs to rely on alternative tissue sources in which self-renewable stem cells could be propagated indefinitely and in a standardized manner. In this way, many alternatives were explored to generate homogeneous DA neurons, using among others, embryonic stem cells (ESCs) [30], neural stem cells (NSCs) [31], induced pluripotent stem cells (IPSs) [32] and adult multipotent stem cells, primarily represented by mesenchymal stem cells (MSCs), such as bone marrow stromal cells [33]. It has been demonstrated that cells with DA properties can be generated from different sources of stem cells and improvements can be seen after implantation of these cells in animal models of PD [34–39]. The accumulated evidence to date, make it difficult to expect a complete recovery of CRT-treated PD patients. Data obtained from animal studies demonstrated that the maximal recovery achieved was approximately 50–60%. This could be related to the implantation site in the striatum, consequently not fully restoring the nigrostriatal pathway. This could also suggest that dopamine may not be the only player needed to restore the affected system in PD. Regarding the implantation site, most of the grafts have been placed in the striatum, which is not the natural site of the DA neurons. Theoretically and according to the residence site of DA neurons, they should be transplanted into the substantia nigra pars compacta area. This procedure carries other major problems as mentioned above, including increased surgical risk and the need for growth of DA neuron projections through the nigrostriatal pathway, which has not yet been effectively achieved.

Currently, two clinical trials involving CRT and PD are recruiting patients, according to the US NIH [201]. These trials are now in Phase I and III and include autologous transplantation into the striatum of bone marrow MSCs [202] and embryonic DA mesencephalic cells [203], respectively. In addition, a clinical trial on IPS generation from somatic cells of PD patients has been recently initiated [204].

Explored cells for CRT

Different cell types have been proposed as viable candidates to generate mature nigrostriatal neurons suitable for CRT in PD. Ideally, grafted DA neurons into the substantia nigra should be able to reconstruct the nigrostriatal pathway, re-establishing the DA modulation of the basal ganglia circuit and promoting regulatory inputs to the DA-secreting neurons that allow compensatory mechanisms, such as feedback [28]. This 'ideal' concept mentioned above is a description of how DA neurons should perform in a best case scenario. This potential scenario can only be achieved through the transplantation of the new cells into the substantia nigra area with the consequent synaptic re-establishment, but could not be achieved through striatal transplantation. The produced DA neurons must show regulated DA synthesis and release, be capable of growth of DA projections through the nigrostriatal pathway in order to innervate the striatum, induce a re-establishment of a dense terminal network throughout the striatum and, of course, a successful integration of the transplanted neurons into the host neural circuitries is needed. Moreover, the produced DA neurons

should exhibit molecular, morphological and electrophysiological properties of substantia nigra neurons, harboring the capacity to reverse motor deficits and be able to survive for decades in the human brain [40]. Several sources of catecholaminergic cells, including autografts of the adrenal medulla and allografts or xenografts of mesencephalic fetal tissue, were successfully assessed in animal models, but their clinical translation has yielded poor results and much controversy. While the fetal tissue trials were ongoing, researchers explored a variety of alternative cell sources of DA neurons, including other animal species, human cadaveric tissue and autologous transplantation. The objective was to overcome the practical and ethical limitations of using human fetal cells for large-scale clinical applications. Porcine mesencephalic DA fetal neurons were grafted into a rat model of PD [41]. The transplanted cells survived and motor improvements have been reported. In addition, a Phase I clinical trial [42] and a similar double-blind placebo-controlled study were performed, but no significant improvements in PD symptoms were documented [42,43]. Mesencephalic fetal porcine cells presented similar challenges to success as human fetal tissue graft, adding the xenograft immunological complications [44]. Human retinal epithelial cells obtained from cadaveric tissue were also tested as candidates owing to the L-DOPA mechanism those cells harbor. Preclinical trials in rats and monkeys through intrastriatal transplantation reported cell survival, as well as motor function improvements [45,46]. A pilot human study showed UPDRS improvements and a double-bind Phase IIb clinical trial was initiated, but was suspended for not reaching the primary study requirements [47]. After the failed adrenal medulla transplantation trials carried out by Backlund [23] and Madrazo [48], more autologous cell sources were explored. Autologous sympathetic ganglion cells grafts showed improvements in transplanted rats [49]; however, in a trial of 35 patients this procedure did not show significant improvements [50]. Carotid cell bodies or glomus type I cells, which are located near the carotid artery bifurcation, are derived from the neural crest and release a variety of neurotransmitters, including acetylcholine, ATP and dopamine [51]. Striatal transplantation of carotid cell bodies was also attempted and showed an improved motor behavior in rats [52] and monkeys [53]; however, a clinical trial that showed initial success did not end with encouraging results [54].

Owing to recent biological breakthroughs associated with advanced technologies, new cell sources, such as stem cells, have emerged (Figure 1). It is important to bear in mind that the concept of the 'perfect' cell source for DA neuron generation might be a utopic concept – that is, each type of stem cell possesses specific advantages and disadvantages. Unique methodologies have been developed in order to coax specific differentiation processes into DA neurons, including genetic manipulation, exposure to a variety of morphogenetic factors or chemical compounds. To date, a host of different cells has been explored in the search for new DA neuron sources. Hereafter, we will review the experiences gained through the years with some of these cells, towards an efficient and secure DA neuron generation.

Neural stem cells

Rigorously defined, adult CNS 'stem cells' exhibit three cardinal features: they are 'self-renewing', with the theoretically unlimited ability to produce progeny indistinguishable from themselves; they are proliferative, continuing to undergo mitosis; and they are multipotent, with the ability to differentiate into neuroectodermal lineages of the CNS [55]. These lineages include a multitude of different neuronal and glial cells subtypes. Multipotent progenitors of the adult brain are proliferative cells with only limited self-renewal that can differentiate into at least two different cell lineages (multipotency) [56–58]. Lineage-specific precursors or progenitors cells are cells that are restricted to one

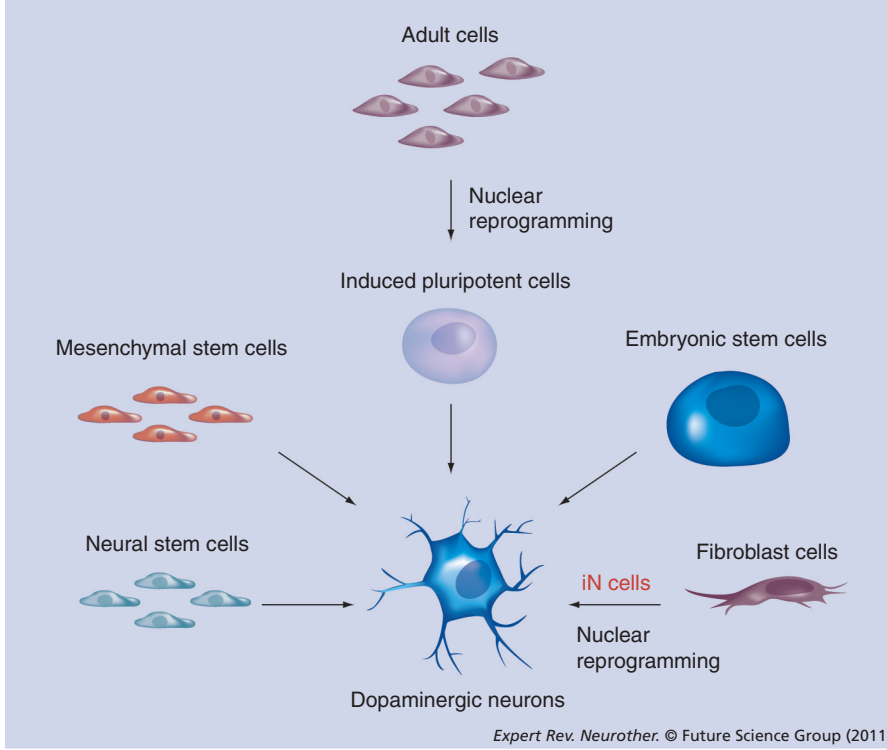

Figure 1. Stem cell sources for dopaminergic neuron generation. Representative scheme of the different stem cells sources explored on the road to dopaminergic neuron generation. To date, dopaminergic neurons have been successfully generated from neural stem cells, mesenchymal stem cells, adult cells through induced pluripotent cells, and embryonic stem cells. Recent discovery of inducible neurons, which directly convert adult fibroblasts into functional neurons [96], may also serve as a suitable source for dopaminergic neuron generation, but no work has been carried out in regard to this objective.
iN: Inducible neuron.

distinct lineage (e.g., neuronal, astroglial or oligodendroglial). Together, CNS stem cells and all precursor/progenitor types are broadly defined as 'neural precursors cells' [55]. NSCs can be found in developing embryos or in the adult CNS in the subventricular zone or in the dentate gyrus in the hippocampus [59]. NSCs have been categorized as multipotent stem cells derived from the CNS with the capacity to regenerate and give rise to cells belonging to all three major cell lineages of the nervous system: neurons, astrocytes and oligodendrocytes [60]. Intuitively, NSCs can be seen as ideal cells for neuron generation, or more specifically for DA neuron generation, since they have the same embryonic origin as the desired DA neurons. For NSC-based CRT in PD, differentiation of these cells into phenotypically stable and functional DA neurons is needed. Fetal rodent and human NSCs have been successfully transplanted into animal models of PD, demonstrating that the implanted cells survive, differentiate and migrate in the host brain. These studies further established that implantation of precursor-derived DA neurons from rodents leads to histological, biochemical and functional recovery in PD animal models [61–65]. Moreover, after long-term expansion and DA differentiation of human midbrain NSCs, no tumor formation has been seen and only mild immunoreactions were observed. Transplantation of rat embryonic VM-derived NCSs differentiated into A9 DA phenotype cells through genetic manipulation and exposure to embryonic VM-tissue explants was able to produce partial striatal reinnervation and a significant restitution of motor function [31]. In regard to adult NSCs, DA neurons were generated by two different protocols. The first one include the DA neuron generation from the subventricular zone NSCs, using the known five-step protocol established in ESCs, and the second one involves the genetic manipulation of adult neural progenitors through Nurr1 ectopic insertion [66,67]. Forced expression of Nurr1, a transcriptional factor specific to midbrain DA neuron development, caused NSC acquisition of the DA neurotransmitter phenotype and sufficient differentiation toward morphologically, phenotypically and ultrastructurally mature DA neurons in adults. The Nurr1-induced DA neurons demonstrated *in vitro* presynaptic DA neuronal functionality, releasing DA neurotransmitter in response to depolarization stimuli and specific DA reuptake [66,67]. Furthermore, Nurr1-engineered adult subventricular zone NSCs survived, and became integrated and differentiated into DA neurons *in vivo*, reversing the behavioral deficit observed in parkinsonian rats [67]. Fetal mesencephalic NSCs and adult NSCs fulfill some important requirements for CRT in PD, such as high yield of functional DA neuron generation starting from a small number of cells, and a major advantage is the fact that they can only be differentiated into CNS cells by natural fate, restricting the cell diversification possibilities [61,68]. Although NSCs seem to be the ideal source for neuronal CRT, their residence deep within the human brain makes them an unlikely source for harvesting. Therefore, researchers looked towards other stem cell population, which are more readily available, with easy access harvesting methods in humans and with plastic functions to generate selective neural populations [69].

Embryonic stem cells

Embryonic stem cells have many characteristics required for an optimal cell source for CRT. ESCs are undifferentiated, self-renewing cells and possess the potential to differentiate into all three germ layers [70]. ESCs taken from the inner mass of the preimplanted blastocyst can be differentiated into NSCs or neural precursor cells (NPCs), and subsequently to DA neurons [32,71]. The DA neurons generated from these cells show morphological, functional and electrophysiological properties of midbrain neurons [37]; however, poor cell survival and phenotypic stability has been observed after intrastriatal transplantation [72]. Undifferentiated ESC were injected into the striatum of 6-hydroxydopamine (6-OHDA) hemiparkinsonian rat model and 14–16 weeks later, 20% of the rats developed teratoma-like tumors, 24% showed no graft survival and 56% had graft-derived, functional, integrated DA neurons within the affected striatum, with behavioral improvements [30]. In order to obviate the potential undesirable side effects derived from contaminated non-DA cells or remaining undifferentiated human ESCs and obtain a homogeneous DA neuronal population, a strict and efficient differentiation protocol is required. Therefore, coaxing human ESCs efficiently into neural lineage is the first step of the DA differentiation procedure [73]. In 2001, three groups separately reported protocols to generate NPCs from human ESCs. The NPCs generated by these methods were able to be further differentiated into cell types of three neural lineages; neurons, astrocytes and oligodendrocytes, in which the NPC-derived neurons were shown to display similar characteristics of mature neurons [74]. Essentially, two main protocols have been established to generate DA neurons from human ESCs: coculture of ESCs with stromal feeder cells [71] and embryonic bodies (EB)-based multistage method [75]. Cultivated human ESCs on PA6 stromal feeder cells resulted in the appearance of TH^+ cells in approximately 87% of the colonies. The generated cells coexpressed DA neuron markers, synthesized and released dopamine and formed a graft stably integrated in the striatum of 6-OHDA-lesioned rats [40]. Utilization of MS5 stromal feeder cells to induce neuronal differentiation, with subsequent treatment with specific factors, yielded a high number of TH^+ cells (30–50% of the cells were neurons and 64–79% of the neurons were TH^+) [76]. On the other hand, the EB-based method has also been used by many laboratories for the differentiation of human ESCs into DA neurons. Recently, Cho *et al.* reported a highly efficient differentiation protocol in which they were able to produce high yield of functional TH^+ cells after differentiation: 77% of the cells differentiated into neurons and 86% of the neurons were TH^+ cells [77]. The most unique feature of this protocol is the generation of neurosphere-like structures, so-called 'spherical neural masses' (SNMs). The SNMs could be expanded for a long time, frozen and thawed freely on demand, and differentiated into DA neurons within 14 days [77]. These characteristics are highly beneficial, enabling the production of a large number of DA neurons within a short time for cell transplantation. Despite all of these promising results, the ESC usage for CRT faces several limitations owing to ethical limitations and their capacity to form teratomas. The problem of controlling cell growth and differentiation is still

unsolved and tumor formation is commonly observed after ESC transplantation [61]. Therefore, several obstacles still prevent the use of ESCs to treat human patients.

Mesenchymal stem cells

Adult stem cell populations isolated from various compartments in the mature organism can display an unexpected plasticity, a process previously thought to be an exclusive feature of ESCs. Subpopulations of adult stem cells are capable of differentiating into mature cells not related to their original lineage, a process termed 'transdifferentiation'. The most well-characterized adult stem cell population considered to possess a transdifferentiation capacity is bone marrow MSCs, also known as mesodermal stromal cells. Most studies on neuronal cell replacement involved the use of MSCs directly, while others differentiated them into neural cell population. In contrast to ESCs or NSCs, MSCs are easy to isolate, can be derived from the patient's own bone marrow and they represent a potential source for autologous cell transplantation, avoiding or reducing immunological rejections. MSCs usage for CRT circumvents the ethical problems concerning fetal tissue usage, thus making them attractive for regenerative medicine research. Despite initial skepticism regarding the capacity of MSCs to differentiate into neurons or glial cells, several studies have established the neurogenic predisposition of these cells as a result of the considerable repertoire of neural gene expression [78–81]. Undifferentiated MSCs transplanted into the striatum of the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD improved mice motor functions and showed double-stained $BrdU^+/TH^+$ cells, indicating that the BrdU-labeled MSCs developed into DA cells *in vivo* [82]. Moreover, recent publications show that the differentiation process of neural-like cells induces the upregulation of genes involved in synaptic transmission, long-term potentialization [78] and the differentiated cells exhibit typical neuronal electrophysiological traits [83–85]. Rodent or human MSCs can be induced to differentiate into TH^+ dopamine-producing cells that appear to be immature neuroblasts of DA lineage, which show weak electrical activity related to low expression levels of voltage-gated ion channels [86,87]. Functional maturity of these induced MSCs improves when the expression of the RE-1 (REST) silencing factor is knocked down by siRNA or by exposure to BDNF [88,89]. Differentiation of MSCs can be induced by exposure to soluble morphogenic proteins (FGFs and sonic hedgehog [SHH]) [39,90], chemical inducers (retinoic acid [RA], dibutyryl cyclic AMP [dbcAMP], 3-isobutyl-1-methylxanthine [IBMX], ascorbic acid [AA] and butylatedhydroxyanisole [BHA]) [39,90] and also by lentiviral transduction of DA transcription factors (LMX1, NURR1 and PITX3) [86]. Transplantation of induced human MSCs into the striatum of 6-OHDA hemiparkinsonian rats, ameliorated behavioral parameters, showing a reduction of 58–80% in apomorphine-induced rotations measured 85, 99 and 125 days after transplantation [39].

Induced pluripotent stem cells

Recent success with the generation of human iPSCs has provided great potential in the stem cell field. Takahashi and Yamanaka developed a technique that is based on the reprogramming of cells from fully differentiated tissues into iPSCs, through viral expression of four transcription factors (termed 'reprogramming factors'): OCT4, SOX2, KLF4 and C-MYC [91]. Although further characterization is needed, iPSCs appear to have similar capabilities as ESCs [61]. This discovery suggested that patient-specific stem cell lines could be generated to study patient-specific disease progression and to treat patients with personalized, tissue-matched transplants without immunological complications [92]. However, a major drawback of the iPSC technology is that it requires viral vectors for reprogramming, which can result in residual transgene expression and may cause malignant tumors. iPSCs have great therapeutic potential for PD and other diseases if safer alternatives for reprogramming can be identified. Recently, Soldner and colleagues reported a novel method for creating iPSCs from skin biopsies of idiopathic PD patients, free of reprogramming factors, using Cre-recombinase excisable viruses [93]. Factor-free iPSCs maintain a pluripotent state and show a global gene expression profile more closely related to human ESCs than to iPSCs carrying the transgenes. This result indicates that residual transgene expression in virus-carrying iPSCs can affect their molecular characteristics and that factor-free iPSCs represent a more suitable source of cells [93]. A study by Wernig *et al.* has shown that mouse fibroblast-derived iPSCs, differentiated into midbrain DA neurons with SHH, FGF8, FGF2 and AA, can functionally integrate in the host striatum of parkinsonian rats and lead to behavioral improvements [32]. However, neuronal overgrowths have been observed, similar to those seen in ESC grafts. The risk of tumor formation by iPSCs is expected to be even higher than that with ESCs, as the reprogramming process involves the regulation of a tumor suppressor gene, *p53* [94]. Another important issue is that patient-derived iPSCs may carry mutations, polymorphisms or epigenetic marks that could make them more susceptible to develop PD-like features [2,70,95]. iPSCs represent a tremendous discovery, but as shown previously, still have many drawbacks and further investigation is needed before clinical application could be attempted. In line with the induced-cell type approach via reprogramming factors, and trying to circumvent the oncogenic potential of the iPSCs, Vierbuchen *et al.* have shown that expression of three transcription factors can rapidly and efficiently convert mouse fibroblasts into functional neurons (inducible neuron [iN] cells) [96]. The iN cells displayed functional neuronal properties, such as the generation of trains of action potentials and synapse formation. Future studies will be necessary to determine whether iN cells could represent an alternative to generate patient-specific neurons [96].

Dopaminergic neuron induction mechanisms

The success of emerging CRT for PD will depend on other issues, such as the implantation site, environment modification or patient selection, the accurate combination of chosen stem cells type and the methodology or differentiation protocol that will be applied. Within a decade, neuroscientists have gathered a wealth of information about the midbrain DA system and have identified many molecular processes and mechanisms that underlie midbrain DA neuron development, maintenance and function. To date, several

protocols have been designed to induce the desired A9 DA neuron phenotype using the abovementioned cells (Table 1). These protocols intend to simulate the natural development process induced by intrinsic and extrinsic factors involved in DA neurogenesis. Within the extrinsic factors, the most widely used are soluble proteins, such as SHH, FGF-2 and -8 and members of the Wnt family. SHH is a morphogenetic factor secreted from the floor plate with a crucial effect on the ventral midbrain during development, and it induces the proliferation of DA neuron precursors [97,98]. In the same way, FGF-8, produced by the isthmus organizer, also plays an important role in DA neuron specification and promotes DA neurogenesis [99]. Members of the Wnt family are additional factors produced and secreted in the midbrain development, which promote activities such as precursor proliferation and neural differentiation [100]. *Wnt* signaling through the action of *Wnt-1*, *-3a* and *-5a* has been reported to be needed for the establishment of the midbrain–hindbrain regions and are involved in activating engrailed (EN) genes, which are necessary for later stages of midbrain DA neuronal development. Moreover, mutant *Wnt* mice showed a loss of most midbrain DA neurons and ectopic expression of *Wnt-1* and *-5a* in NSCs lead to DA differentiation and NURR1 positive cells [101–103]. Other growth factors, such as BDNF, GDNF, EGF and TGF-β, have been used and have shown a DA neuron inducer activity [90]. Chemical inducers are

Table 1. Stem cells and differentiation protocols.

Stem cells	Differentiation factors	Graft survival (weeks)	Behavioral improvement	Ref.
NSC				
Fetal progenitor cells	EGF, FGF-2	20	Yes	[24]
Fetal VM NSC	FGF-2, BDNF, GDNF, NT4/5, SHH	12	Yes	[25]
Fetal VM NSC	FGF-2, FGF-8b, SHH, tgWnt5a	11	Yes	[26]
Fetal VM NSC	FGF-2, FGF-8b	9	Yes	[27]
SVZ aNSC	FGF-8, SHH	ND	ND	[28]
Cograft VM explant/NSC	VM explant coculture, tgPitx3	ND	Yes	[10]
SVZ aNSC	NT-3, BDNF, tgNurr1, tgMash1	6	Yes	[29]
ESC				
D3	*In vivo* differentiation	16	Yes	[9]
R1	FGF-2, FGF-8b, SHH, tgNurr1	8	Yes	[16]
CCE or EB5	PA6 stromal feeder	2	ND	[33]
R1, E14.1, B5	FGF-2, FGF-8b, SHH	ND	ND	[34]
WA09, WA01	FGF-2, FGF-8, SHH, coculture with telomerase-immortalized human fetal midbrain astrocytes	14	Yes	[35]
H1, H7	EGF, FGF-2, IGF-1, PDGF, NT-3, BDNF	ND	ND	[37]
H1, H9, HES-3, R366, Cyno1	FGF-8, SHH, BDNF, GDNF, TGF-β3, AA, dbcAMP, coculture with MS5 stromal cells (tgWnt1 and nontg)	ND	ND	[41]
SNUhES1, SNUhES2, SNUhES3	FGF-8, SHH, AA	ND	ND	[77]
MSC				
BM MSC	FGF-2, EGF, BHA, DHA, dbcAMP, RA, IBMX	18	Yes	[18]
BM MSC	*In vivo* differentiation	4	Yes	[43]
BM MSC	FGF-2, FGF-8, SHH, tgLMX1a	ND	ND	[51]
BM MSC	SHH, FGF-2, FGF-8, BDNF	ND	ND	[54]
iPSC				
O9	(OCT4, SOX2, c-MYC, KLF4); MEF feeder, FGF-2, FGF-8, SHH	4	Yes	[11]
Dermal fibroblasts from PD patients	(OCT4, SOX2, c-MYC, KLF4); MEF Feeder/Ebody, FGF-2, FGF-8, SHH	ND	ND	[93]

Summary of the different stem cells used, their respective differentiation protocols, as well as clinical important properties of dopaminergic stem cell grafts in animal models of PD.
Transgene expression is shown as (tg) followed by the name of the expressed gene.
aNSC: Adult neural stem cell; BM: Bone marrow; ESC: Embryonic stem cell; iPSC: Induced pluripotent cell; MSC: Mesenchymal stem cell; ND: Not demonstrated; NSC: Neural stem cell; SVZ: Subventricular zone; tg: Transgene; VM: Ventral mesencephalon.

also considered to be extrinsic factors that enhance DA neuron formation. Among these factors, RA, dbcAMP, IBMX, AA and BHA are currently the most effective inducers used [39,90,104]. To certain stem cell sources, extrinsic induction can be sufficient to generate DA neurons, but in cases of stem cells that are not naturally prone to become midbrain DA neurons, this process seems to be more difficult, inefficient and incomplete. Induction by intrinsic factors has facilitated and improved DA neuron generation. Genetic manipulation, mainly by lentiviral transduction, has demonstrated that ectopic insertion of transcription factors represented by homeodomain proteins, proneural genes and genes involved in epigenetic control, effectively induce a DA neuron phenotype [68]. The development of mice deficient in *PITX3*, *LMX1A/B*, *EN1*, *EN2*, neurogenin 2 (*NGN2*) and the orphan nuclear receptor 1 (*NURR1*) has facilitated the development of a map that specifies gene–function relationships during midbrain DA neurons differentiation [105]. The expression of the gene coding for LIM homeobox transcription factor 1 (*LMX1A*), has been reported to be both necessary and sufficient for the induction of the midbrain DA phenotype in midbrain neuroepithelial cells, ESCs and MSCs, an effect that can be enhanced by extrinsic factors [86,106]. Moreover, the neurotransmitter phenotype is partly determined by the transcription factor NURR1, which regulates several proteins that are required for dopamine synthesis and regulation, such as TH, vesicular monoamine transporter 2 (VMAT2), dopamine transporter (DAT) and RET receptor tyrosine kinase (cRET) [107,108]. Overexpression of PITX3 and FOXA2, other transcription factors involved in DA specification, was seen to actively assist NURR1 and LMX1A to induce human ESC and NSC terminal maturation to midbrain DA neurons [109–111]. Currently, most protocols rely on early induction through SHH, FGF-8, WNT-1/-5A, TGF-β and RA, which are often combined with the introduction of transcription factors, such as LMX1A and NURR1. The generated cells should be monitored for the expression of NURR1, EN1, EN2 and the midbrain DA neuron-specific gene *PITX3*, which display the full DA phenotype. In order to achieve rapid and safe clinical translation, transgenesis must be preferably avoided until safer gene delivery methods can be developed.

Clinical CRT trials in PD

Initial experiments of DA cell transplantation in human PD patients began three decades ago. The first clinical trials used adrenal medullary or fetal VM allografts (transplants between genetically dissimilar individuals within the same species) transplanted into the striatum as a source for DA neurons [48,112,113]. These first studies did not show significant improvements; however, several open-label clinical studies that followed demonstrated encouraging results [114–121]. The first human studies were carried out on a small number of patients and, despite cautious optimism, the improvements found were modest [23,24,48]. Several studies followed demonstrating a wide variability of patients' outcomes. Some exhibited marked improvement, in some cases even permitting withdrawal of antiparkinsonian medication; others showing modest improvements, and others did not display any benefit. Motor aspects as quantified by the motor part of the UPDRS were the major outcome measured, while non-motor features of PD were not investigated. Motor improvements, when they occurred, had a positive impact on activities of daily living and quality of life [114–119]. Tremor and postural instability improved least, similar to the poor response of these symptoms to L-DOPA therapy. The clinical improvements reported by these open-label neural grafting trials have been long-lasting. Post-mortem studies undertaken in grafted patients that have died demonstrated survival of grafted fetal VM DA neurons with local reinnervation of the striatum by these cells for long periods postoperatively [120,122]. Functional imaging by F-DOPA PET also demonstrated that the grafted patients had increased fluorodopa uptake [123,124]. Such studies have suggested that approximately 100,000 DA neurons need to be present within the grafted striatum to achieve significant clinical benefit, and that those of nigral origin are most competent in innervating the striatum [125].

Two double-blind placebo-controlled trials of fetal VM transplantation in PD followed these promising initial studies. The first of these controlled trials was published in 2001 by Freed *et al.* and included 40 PD patients aged 34–75 years, suffering from advanced disease (mean disease duration: 14 years) [29]. Patients were randomly assigned to receive a VM transplant or sham surgery – a burr hole with no penetration of the dura – thereby achieving blindness of the patient and the assessing neurologist. Transplants included VM of two embryos (7–8 weeks old) and were transplanted into the putamen on both sides of the patient's brain. VM tissue was cultured 1–4 weeks prior to transplantation and unusually, prepared into strands of tissue [126]. Immunosuppression was not given, unlike the open-label studies that had all used standard immunotherapy [29]. The primary outcome, which was a change in a subjective global rating of clinical improvement at 1 year post-transplantation, did not reveal significant improvement [29]. Significant improvements were seen on UPDRS motor scores in 'off' times in grafted patients who were younger than 60 years of age. However, subsequent analysis suggested that the main determinant of this improvement was the preoperative L-DOPA responsiveness rather than the patients' age. Ma *et al.* reported the long-term outcome in 33 of the original trial participants who were followed for 2 years after transplantation and 15 of these subjects who were followed for 2 additional years [127]. They claimed that a high residual preoperative level of dopamine in the anterior putamen, as determined by F-DOPA PET, was associated with better clinical outcome [127]. PET scanning showed significant increases in F-DOPA uptake in the putamen of transplanted patients and post-mortem examinations showed DA neuronal survival and fiber outgrowth in the grafts. These results suggest that clinical benefit and graft viability are sustained up to 4 years after transplantation. Moreover, the dependence of clinical (but not imaging) outcomes on subject age and sex at 1 year may not persist over the long term. However, these results must be cautiously interpreted since the patient number was not large enough, longer follow-up duration is needed and the symptomatic benefit was smaller than expected by PET results. Moreover, the number of surviving grafted dopamine neurons

was low compared with those previously reported in open-label studies [29]. The main motor improvements were similar to those seen with DA medications and included rigidity and bradykinesia, while tremor did not change.

The second double-blind sham surgery controlled trial [128] involved 34 patients with advanced PD, aged 30–75 years. Patients were randomized to receive either bilateral transplants (from one or four donors in each side) or sham surgery. The surgical technique differed from the previous study, solid pieces of VM tissue were obtained from 6–9-week fetuses, stored in a hibernation medium for 2 days and surgery was performed by two-stage procedure separated by a week. All patients received immunosuppression with cyclosporine for 6 months postoperatively [128]. Primary outcome was motor component of the UPDRS in 'off' state, between the baseline and the final 24-month visit. Although there was a clear trend for benefit, it did not reach statistical significance. *Post hoc* analysis demonstrated significant motor benefits in patients suffering from milder disease [128]. Importantly, patients in both the one- and four-donor transplant groups showed significant motor improvement compared with placebo at 6 and 9 months post-transplant, but not thereafter. It was speculated that discontinuation of the immunosuppressive therapy triggered an immune response to the graft, resulting in loss of transplant function. Indeed, the magnitude and time course of the initial improvement (up to 6–9 months) was similar to that reported in earlier open-label studies [112,121,129]. Despite a possible immune system-mediated impairment in graft function, PET scanning showed significant bilateral increases in striatal fluorodopa uptake in transplanted groups. Post-mortem data from this study also showed that the DA neurons survived in large numbers (~100,000 per putamen in the four-donor group and 30,000 in the one-donor group), with marked reinnervation of the striatum [128]. These findings are surprising and the explanation that the clinical benefits were transient owing to immune mediated graft impairment does not settle well with the increased striatal uptake demonstrated by PET and the post-mortem findings. It seems contradictory to hypothesize that an immune response reduced the function of the graft after 9 months and on the other hand to observe significant increases in F-DOPA signal at the end of the study and significant reinnervation of the striatum at autopsy, therefore, the immune involvement should only be viewed as speculation.

These studies proved the concept that transplanted DA neurons could survive in the brains of PD patients, become functionally integrated and suggest persistent clinical improvement. Nevertheless, the studies showed that fetal VM transplants produced very variable responses. The reason for this variability was not clear, and possible explanations include technical issues, such as tissue preparation, implantation procedures, or patient selection variables, such as age, levodopa response or disease stage [125].

One of the big questions about CRT for PD is why all of the encouraging results obtained in animal models have failed to be translated in human clinical trials? Several possible explanations could explain these phenomena. First, experimental models always only partially recapitulate the disease. The animal models in which experimental CRT were tested, such as 6-OHDA in rats or MPTP in monkeys, consist of the administration of a neurotoxic substance that, through an acute injury, produces massive DA neuron death. Yet, PD pathology in humans is not an acute pathology, but a progressive chronic degenerative process occurring in the CNS. Second, the environment that the transplanted cells are exposed to differs in the diseased brain and in animal models brains. Chronic diseases, in particular neurodegenerative diseases, such as PD, present a highly deranged environment, which includes, among others, heavy oxidative stress, protein aggregation and trophic support deficiencies induced by malfunction of neuron support cells. These mechanisms are less prominent in animal models. Third, most transplanted patients suffered from long-lasting severe disease, therefore a selection of better suited patients could ameliorate the results. These differences are not trivial and might be of major importance when trying to translate a therapy developed in animal models to humans. Therefore, encouraging results from animal models prompt experiments in humans patients; however, expectations should be limited and defining the best suited patient for the therapeutic intervention is imperative.

Graft-induced dyskinesias

This failure of CRT to show clear benefit in the primary outcome parameters in the double-blind studies was disappointing. In itself, this would have been discouraging, but of greater concern was the description of the development of significant graft-induced dyskinesias (GIDs). GIDs are involuntary movements – dyskinesias – that occur in the absence of medication, but in the presence of the graft. Freed *et al.* reported GIDs in 15% of the transplanted patients more than 1-year post-transplant. Several of these patients required further surgical intervention with subthalamic DBS to relieve them of these troublesome GIDs [130]. A PET study showed DA hotspots, especially in patients developing GID [127]. Although these hotspots were not described in the PET results from longer follow-up times [127], the mode of cell preparation and graft implantation procedure by Freed *et al.* was speculated to contribute to the development of GIDs.

However, the second placebo-controlled study by Olanow *et al.* also reported the development of significant 'off-medication' GIDs in 56.5% of the grafted patients 6–12 months after transplantation [128]. These GIDs typically consisted of stereotypic, rhythmic movements of one or both lower extremities, with three patients requiring further surgical intervention to reduce their severity.

Importantly, GIDs were only described in patients who previously suffered from L-DOPA-induced dyskinesias, yet without correlation to their severity [125]. Animal studies also demonstrated that L-DOPA priming is required for GIDs [125].

Several theories from different laboratories around the globe are trying to explain the causes of GID. Several mechanisms have been proposed; however, the pathogenesis of this type of dyskinesia remains unclear and there is no effective way to avoid this complication, nor an effective simple treatment.

The first theory of the origin of GID was that it stemmed from imbalanced DA innervation. It has been suggested that GIDs developed as a result of fiber outgrowth from the graft, causing increased DA release [29] or as a result of imbalanced DA reinnervation [131,132]. Ma *et al.* reported follow-up and imaging results on five transplanted patients with GID who were no longer taking DA medications [127]. In this study, these subjects all belonged to the young subgroup of transplant recipients and had greater clinical improvement and putamen F-DOPA uptake at 1 and 2 years than those who did not develop GID. However, Hagell *et al.* reported no differences in either regional or global levels of striatal DA reinnervation between transplanted PD patients that presented GID to those that did not develop GIDs [133]. Another study reported that no correlation was found between the presence of GID and an excessive DA reinnervation [128]. Other theories obtained from animal models proposed that GIDs could occur as a failure of the graft to restore DA synaptic contacts with the host striatal neurons, resulting in abnormal signaling and synaptic plasticity [134]. Immunological implications have also been proposed in which inflammatory responses are triggered against the graft [128]. This is in line with the clinical observation that GIDs occurred after early discontinuation of immunosuppressive therapy, with signs of inflammatory reactions around the graft, as seen in autopsied subjects [128,135]. As stated by Politis *et al.*, an immunological reaction around the graft may cause a degree of tissue rejection, reducing the restoration of striatal synaptic DA levels, which can be related to GIDs [132].

Another theory is that GIDs are the consequence of serotonergic (5-HT) neurons cografted in these transplants and engage in nonphysiological properties, such as false transmitter release. Serotonergic neurons are present in the developmental stages of the VM, and therefore they are also transplanted with the graft. Since 5-HT neurons are physiologically able to store and release DA, GIDs can occur as a result of DA level mishandling. The hypothesis proposes that 5-HT neurons are responsible for dysregulating the DA release in the synapse, as a result of graft-derived excess of 5-HT neurons interacting with the normal DA neurons [132,136].

Using *in vivo* brain imaging, Politis *et al.* showed excessive serotonergic innervation in the grafted striatum of two patients with PD who had exhibited major motor recovery after transplantation with dopamine-rich fetal mesencephalic tissue, but had later developed off-medication dyskinesias [136]. Moreover, the dyskinesias were significantly attenuated by administration of a serotonin agonist, which activates the inhibitory serotonin autoreceptors and attenuates transmitter release from serotonergic neurons [136]. These results indicate that GIDs were caused by the dense serotonergic innervation engaging in false transmitter release. Finally, another theory claims that PD patients that previously suffered from dyskinesias are more prone to develop GID owing to a priming effect.

Graft-induced dyskinesia presents a major problem in CRT for PD since this is a critical side effect that necessitated further surgeries in some of the patients. Moreover, it may limit the amount of cells that could be transplanted. Possible solutions to the problem include using tissue free of other cell types (such as serotonergic neurons) or only selecting patients for grafting who do not suffer from dyskinesias.

Does PD affect the grafts?

In order to answer this question, post-mortem studies from long-term implanted patients were needed. Indeed, reports concerning this issue have only recently emerged. Mendez *et al.* reported no PD pathology in a graft that survived for 14 years [137]. Several patients that were transplanted 4–9 years before their death did not contain any PD pathology in the grafted cells [40,95]. However, Li *et al.* reported two patients transplanted twice, who died 13–16 years after their first transplantation. They had numerous surviving DA neurons, some of whom contained α-synuclein and ubiquitin-positive Lewy bodies and neuritis [138]. In a second report, Kordower *et al.* described two patients that died 14 years after transplantation who showed aggregated and neurotic α-synuclein, as well as decreased staining for dopamine transporter with normal staining for TH and VMAT-2 [139]. Clinically, these patients suffered from a progressive clinical deterioration from 11–12 years post-transplantation. These findings may suggest progressive graft failure after a decade with compensatory changes [139]. Li *et al.* recently reported on a 12-year-old graft with 1.9% of the DA neurons containing Lewy bodies and a 16-year-old graft with 5% of the cells containing Lewy bodies from two autopsies [140].

Taken together, these are encouraging reports since they suggest that transplanted cells may integrate and function for more than a decade and most cells will still not be affected by the diseased brain in which they are implanted in. Moreover, it is yet to be explored whether DA cells from different origins are as vulnerable to the disease process as the previously reported embryonic DA neurons.

Conclusion & perspectives

Regenerative medicine has an extraordinary potential to ameliorate the quality of life of PD patients and offers a possible cure for PD. The initial clinical trials in CRT for PD employed embryonic mesencephalic grafts, and provided proof of principle that DA cell replacement can be achieved and, in selected patients, can accomplish significant, long-lasting improvement of motor function. On the other hand, the excitement generated by the early open-label trials were diminished by the subsequent placebo-controlled, double-blind trials. Nevertheless, accumulated data and several fascinating breakthroughs in stem cell research created an opportunity for better and safer CRT options (e.g., transplantation of autologous bone-marrow-derived stem cells). In the previously decades, our basic knowledge about stem cells and the potential CRT for PD has grown exponentially, as seen by increasing scientific publications. A vast progress was obtained in generating human-derived DA neurons from different stem cell sources. Specifically, we achieved one of the first milestones for CRT, which consists of producing large quantities of standardized human stem cell-derived midbrain DA neurons *in vitro*. However, their efficient and safe application in animal models of PD has not yet been achieved. As stated by Arenas *et al.*, in order to advance on the CRT pathway, we need to:

improve protocols for the generation of midbrain A9 DA neurons; identify markers and develop protocols for the separation of transplantable cells; eliminate any chance of tumor formation or neural outgrowth; prevent excessive inflammatory response; improve imaging methods to monitor graft and DA cell function *in vivo*; improve animal models of PD to recapitulate more features of the disease and increase predictability; eliminate the response from the host to xenografts or increase tolerance; and comply with Good Manufacturing Practices and the increasing regulatory requirements [2]. There is a long way to go in order to successfully translate the generated knowledge into safer clinical applications. Currently, two PD–CRT clinical trials are in progress. These long-expected trials are currently in Phase I and III and include autologous bone marrow MSC transplantation [202] and embryonic DA mesencephalic cell transplantation [203], respectively. Multidisciplinary research must be carried out, combining basic and clinical research, in order to step forward on the road of CRT in PD.

Expert commentary

Cell replacement therapy for PD was proposed three decades ago as a possible approach to treat PD. During this time, many milestones have been successfully reached, creating a lot of hope and excitement. It is now established that cells can be transplanted into the brain, integrate and survive for years, and release dopamine. Clinical studies demonstrated the feasibility of cell transplantation in human PD patients, demonstrating a proof of concept that CRT for PD can be possible.

Although positive results were obtained, several obstacles have kept this therapeutic approach away from the clinic. DA neuron production for replacement must be refined in order to produce 'pure' DA neurons and eliminate the danger of tumor production, as well as synaptic dysregulation and side effects. It is also crucial to elucidate where and how to graft these cells in order to amend the diseased DA system in PD. Most studies transplanted the cells in the striatum, which is not the natural place for DA neurons. The natural place for DA grafts would be the substatia nigra pars compacta, but implantation at this site raises severe surgical and physiological problems, including the feasibility of artificial reconstruction of the nigrostriatal pathway.

It is difficult to foresee complete motor improvement using the current CRT protocols, mainly because of the inability to fully restore the natural nigrostriatal pathway and the hostile environment in which the cells are transplanted. Nevertheless, protocol refinements in parallel with implantation improvements and environment-modifying therapies are expected to yield better results and enable the clinical utilization of CRT in PD.

Five-year view

Cell replacement therapy carries great potential for future medicine and it is expected that this strategy could aid in treating many devastating illnesses. With the fast growing technological breakthroughs and the physiological dissection of both stem cell and DA neuron developmental pathways, it is hoped that CRT in PD could reach clinical use in 5 years time. Currently, two clinical trials of CRT for PD have started and these will probably produce guidelines for future strategies in this long awaited field. More sophisticated cells with higher differentiation efficiencies and better safety will be generated from different stem cell niches and IPSs. Tissue engineering developments may contribute to the graft cell procedures, enhancing survival and promoting cell protection against a hostile diseased environment. Environment-modifying therapies will be developed, probably through the

Key issues

- As 'proof of principle', it was demonstrated by several clinical studies performed in the 1990s that replacement of lost dopaminergic neurons in the striatum can improve motor symptoms in animal models and Parkinson's disease (PD) patients.
- Open-label clinical studies using fetal ventral midbrain tissues demonstrated encouraging results with long-term survival of grafted fetal dopaminergic neurons and local reinnervation and increased fluorodopa uptake.
- These studies have suggested that approximately 100,000 dopaminergic neurons need to be present within the grafted striatum to achieve significant clinical benefit, and that those of nigral origin are most competent in innervating the striatum.
- Long-term evaluation of grafted cells in post-mortem PD patients have reported that some of the transplanted cells contained α-synuclein, ubiquitin-positive Lewy bodies and neuritis.
- Double-blind placebo-controlled trials of fetal ventral midbrain transplantation in PD suggested that transplanted dopaminergic neurons could survive in the brains of PD patients, become functionally integrated and suggested possible clinical improvement. Nevertheless, these clinical trials did not reach successful results.
- Two clinical trials involving cell replacement therapy and PD are now being performed. These trials are now in Phase I and III and include autologous transplantation into the striatum of bone marrow mesenchymal stem cells and embryonic doaminergic mesencephalic cells, respectively. A clinical trial format on induced pluripotent stem cell generation from somatic cells of PD patients has been initiated recently.
- Owing to new advancements in biology, other cell sources, such as neural, embryonic, mesenchymal and induced pluripotent stem cells, are being investigated.
- Currently, most protocols rely on early induction of cell sources through sonic hedgehog, FGF8, WNT1/5A, TGFβ and retinoic acid, which can be combined with the introduction of genes codifying for transcription factors, such as LMX1A and NURR1.
- The success of emerging cell replacement therapy for PD will reside among other issues, such as implantation site, environment modification or patient selection, the accurate combination of chosen stem cells type and the methodology or differentiation protocol that will be applied.

cotransplantation of trophic cells that could improve the cellular milieu, enhancing the function and establishment of the new implanted graft. Cell engineering might also contribute to CRT, developing a dopamine secreting cell, where secretion could be regulated trough inducible mechanisms. This could alleviate some of the graft-induced secondary effects produced by focally and constitutive secretion of dopamine. On the other hand, the great promise of regenerative medicine and the excitement of the stem cell research field has generated huge hope and expectations for PD patients, creating an economic opportunity in which scientists and clinicians must nurture countries' public administration policy makers.

Financial & competing interests disclosure

This work was performed in partial fulfillment of the requirements for a PhD degree for Javier Ganz. This work was supported by the Devora Eleonora Kirshman Fund for research of Parkinson's Disease, Tel Aviv University (Eldad Melamed), by the Norma and Alan Aufzein chair of research of Parkinson's Disease (Eldad Melamed) and the Israeli Science Foundation (ISF; Nirit Lev). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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