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Immortalization of neuronal progenitors using SV40 large T antigen and differentiation towards dopaminergic neurons

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Abstract

Transplantation is common in clinical practice where there is availability of the tissue and organ. In the case of neurodegenerative disease such as Parkinson's disease (PD), transplantation is not possible as a result of the non-availability of tissue or organ and therefore, cell therapy is an innovation in clinical practice. However, the availability of neuronal cells for transplantation is very limited. Alternatively, immortalized neuronal progenitors could be used in treating PD. The neuronal progenitor cells can be differentiated into dopaminergic phenotype. Here in this article, the current understanding of the molecular mechanisms involved in the differentiation of dopaminergic phenotype from the neuronal progenitors immortalized with SV40 LT antigen is discussed. In addition, the methods of generating dopaminergic neurons from progenitor cells and the factors that govern their differentiation are elaborated. Recent advances in cell-therapy based transplantation in PD patients and future prospects are discussed.

Keywords: SV40 large T antigen • neuronal progenitors • dopaminergic neurons • Parkinson's disease • immortalized cell lines • stem cells • transplantation.

Introduction

Transplantation of organ and tissue are common in clinical practices for treating chronic diseases. In case of neurodegeneration in the central nervous system (CNS), organ transplant is not possible and hence, cell therapy is an innovation in clinical practice. Transplantation of progenitor's cells with differentiation properties or differentiated cells is a promising approach in treating neurological diseases such as Alzheimer's type dementia, Parkinson's disease (PD), Huntington's disease (HD), stroke and trauma [1, 2].

Parkinson's disease is one among the common neurodegenerative disorders, caused by the degeneration of nigrostriatal dopamine neurons. Transplantation of cell and tissue has been developed as a clinical approach for treating PD [3–10]. Nevertheless, the treatment

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is limited to the availability of donor tissue. One of the alternative cell sources for transplantation could be immortalized cell lines [11]. It has been shown that transplantation with conditionally immortalized progenitor's cells could be useful in treating PD [12, 13]. Immortalized cell lines also helps in elucidating the mechanism of cellular differentiation.

There are several ways (Table 1) to immortalize primary neuronal cells namely somatic fusion [14], v-myc [15], SV40 large T antigen

Table 1 Immortalization of neuronal cells using various agents

Immortalizing agent	Cell line	Characteristic features	Transplantation experiments	Reference
Somatic fusion [hypoxanthine phosphoribosyltransferase-deficient neuroblastoma cell line (N18TG2)]	E14 mouse rostral mesencephalic tegmentum (MN9D)	Express neurofilament		Choi *et al.* [14]
c-myc	E11 mouse embryonic midbrain (A1)	Express vimentin and nestin, MAP, co-stained for GFAP and NSE No TH and DAT mRNA expression Glutamic acid decarboxylase mRNA has been observed		Colucci-D'Amato *et al.* [173]
c-mycER^{TAM} (c-myc protein fused with a mutated oestrogen receptor)	Human ventral mesencephalic cells 10 week old aborted foetus)	Nestin positive, differentiate TH *in vitro*	Rodent PD model: Improved behavioural recovery, no TH differentiation *in vivo*, but increased host TH immunoreactivity	Miljan *et al.* [174]
v-myc	Human ventral mesencephalic cells (10 week old aborted foetus; hVM1)	Express Lmx1A, Lmx1B, Girk2, ADH2, Nurr1, Pitx3, VMAT2 and DAT, βIII-tubulin and TH	Transplantation of hVM1-Bcl-XL in Hemiparkinsonian rats: No tumour formation, integrated into host parenchyma, expresses TH, DAT Apomorphine-induced rotation was not compensated, while amphetamine-induced rotations were compensated [175]	Villa *et al.* [176], Tonnesen *et al.* [177]
	Human ventral mesencephalic cells (8 week-old aborted foetus; MESC2.10)	Proliferation: Express Nurr1 and GFR α1 Differentiation: Express TH, GFR α1 and c-ret mRNA increased	Hemiparkinsonian rats: No TH expression, no behavioural recovery in amphetamine-induced motor asymmetry test	Paul *et al.* [178]
Telomerase (hTERT)	Human foetal subventricular zone (hNPC-TERT)	Proliferation: Co-express nestin and GFAP Differentiation: Express MAP2, O1 and GFAP *i.e.* differentiate into neurons, oligodendrocyte and astrocytes respectively)	Spinal injury models: Recovery of motor functions and electrophysiological parameters [179]	Bai *et al.* [180]

(LT) [16, 17] and human telomerase [18]. The commonly used vector for immortalization is SV40 LT, which exhibits immortalizing properties without fully transforming the cells [19]. The focus of this review is on the cell lines established from mesencephalic progenitors, as it is shown *in vivo* that mesencephalic progenitors differentiate into dopaminergic neurons. The article attempts to discuss the mechanism of generating differentiated dopaminergic neurons and their application for transplantation, breaking them down into three divisions: (1) the molecular mechanism of transformation by SV40 LT and discusses the factors involved in immortalization, (2) the influences of genes in differentiation of dopaminergic neurons and the characteristic features of SV40 immortalized neuronal cell lines with respect to dopaminergic differentiation, (3) transplantation studies and the limitation for cell therapy with stem cells in PD patients.

History of SV40 LT antigen and its mechanism in immortalization

Discovery and molecular mechanism of SV40 LT antigen

The simian virus SV40 was first discovered in 1960 [20] in cultures of rhesus monkey kidney cells that were being used to produce polio vaccine. It was named for the effect it produced on infected cells, which developed an unusual number of vacuoles. This led to the discovery of tumour formation by SV40 in rodents as well as induced transformation of primary cultures of human cells [21, 22].

The simian virus SV40 is a double stranded DNA virus with a genome of 5243 base pairs, belonging to the family *Polyomoviridae* [23–25] and its natural host is the rhesus species (*Macaca mulatta*) [26]. The genome codes for seven proteins, three structural and four functional proteins in overlapping reading frames [23, 24]. The structural proteins are VP1, VP2 and VP3 and the functional proteins include a large T antigen and a small t antigen essential for viral life cycle and two small proteins of unknown function namely, agnoprotein and 17kT [25, 27, 28]. The simian virus SV40 large T antigen is a multifunctional regulatory protein [23], classified as a member of helicase superfamily with the property of unwinding double stranded DNA and RNA [29–31]. It encodes 708 amino acids and consists of J domain, Rb-protein binding (LxCxE) motif, nuclear localization signal domain, origin binding domain, Zn domain, ATPase domain, variable region and host range (HR) domain.

Role of LT antigen in transformation/immortalization

The transformation/immortalization by SV40 LT involves the following mechanism(s): (1) activation of E2F-mediated transcription through binding with Rb-E2F complex and (2) inhibition of p53, by blocking p53-dependent transcription activation and p53-independent growth-arrest. These two mechanisms lead to overcome growth arrest, prevent apoptosis and result in cellular proliferation.

Interaction of LT antigen with Rb protein—The LT binds to Rb protein through the LxCxE motif along with J domain [27, 29, 32]. The J domain of LT has sequence similarity with the J domain of DnaK class of molecular chaperones [33]. Large T antigen binds in an ATP dependent manner to the hsc70 (DnaK homologue present in mammalian cells), and the binding is dependent on J domain [28]. The binding of LT to Rb protein suppresses the pathway of cell cycle entry and growth arrest, which are governed by Rb-E2F complexes. E2F, a transcription factor along with Rb controls the transcription of E2F-regualted genes, which encode proteins required for DNA replication, nucleotide metabolism, DNA repair and cell cycle progression. The disruption of the repressive effect of Rb-E2F complex by LT results in the transcription of E2F dependent genes and progress into S phase; thereby cells are transformed to proliferate continuously.

Interaction of LT antigen with tumour suppressor p53—In SV40 transformed cells, LT was found to bind with p53 [34]. p53 is a transcriptional activator that mediates apoptosis under unfavourable conditions like DNA damage, depletion of nucleotides and abnormal inhibition of Rb protein. The ATPase domain of LT helps in the binding with p53 protein [35]. The carboxy-terminal variable region and HR domain (amino acid 351–708) of LT are not required for p53 binding, but it requires the regions 351–450 and 533–626 amino acids, known to be the bipartite region for the interaction with p53 [36]. In general, binding of LT with p53 is responsible for an extended life span and cellular transformation, by blocking p53-dependent transcription activation and p53-independent growth-arrest [29, 37, 38]. Binding of LT to p300/CBP that interacts with p53 also prevents apoptosis and leads to the survival of the cell [39].

SV40 LT antigen immortalized neuronal cell lines

The SV40 LT has been widely used in the production of various cell lines (Table 2). However, limited studies have focused on the immortalization of mesencephalic progenitor cells towards dopaminergic/neuronal phenotype.

Immortalization with SV40 LT antigen

$1RB_3AN_{27}$/N27 cell lines—The establishment of dopamine-producing immortalized clones was first reported by Prasad *et al.* [16]. The cells were established by transfecting rat E12 primary mesencephalic cells with pSV_3neo vector expressing SV40 LT and were found to be positive for tyrosine hydroxylase (TH) expression. The clones derived from pSV_3neo transfected cells were subcultured and the subclones were found to contain over 95% of TH-positive cells ($1RB_3AN_{27}$). These $1RB_3AN_{27}$ cells produced homovanillic acid, a metabolite of dopamine [16] and exhibited dopaminergic properties with the expression of DAT and TH. In addition, these cells showed little or no labelling with GFAP, which is a marker for astrocytes [40].

Immortalized VMP E12 neuronal progenitor (iVMP) cell lines—The neuronal progenitor cells from the rat mesencephalon were isolated from 12th day of embryogenesis (E12) and were non-virally transfected with pSV_3neo vector expressing SV40 LT [41]. Four

Table 2 SV40 large T antigen immortalized neuronal cell lines.

Immortalizing agent	Cell line	Cell line derived from	Properties of the cell line	Reference
SV40 large T antigen	$1RB_3AN_{27}$	E12 rat primary mesencephalic cells	Express SV40 LT, TH, homovanillic acid	Prasad *et al.* [16]
			Express DAT, neuron-specific enolase, nestin	Adams *et al.* [40]
	iVMP (C1, C2, C3 and C4)	E12 rat ventral mesencephalic cells	Proliferation (C2-C4 clones): Express Lmx1, Wnt1, Wnt5, Nurr1, Dlk1, En1, SV40 LT Differentiation (C2 and C3 clone): Lmx1, Wnt5, Nurr1, En1, Dlk1, Ngn, Pitx3, DAT, TH C1 clone: βIII-tubulin immunopositive after differentiated by SV40 LT silencing No TH immunoractive cells were observed	Nobre *et al.* [42]
N-terminal fragment of SV40 large T antigen (T155)	AF5 (T155g)	E14 rat primary mesencephalic cells	Express SV40 LT, neuronal and astrocytic markers	Truckenmiller *et al.* [17]
			Express TH, βIII tubulin, growth factors PDGF, TGF β1, TGF β2, neurotrophic factors GDNF, BDNF and bFGF	Truckenmiller *et al.* [43]
			Express Pitx2, GABA	Sanchez *et al.* [44]
	RTC3/4 (T155c)	E14 rat primary mesencephalic cells	Immunopositive for S100β and vimentin, negative for NeuN, MAP2 and βIII-tubulin. Secrete PDGF	Harvey *et al.* [181]
Temperature sensitive SV40 large T antigen	CSM14.1	E14 rat primary mesencephalic cells	Proliferation at 33°C—express SV40 LT, Nurr1 and nestin Differentiation at 39°C—no expression of SV40 LT, express MAP2, Nurr1, TH, ADH2	Hass and Wree [45]
	SN4741	E13.5 transgenic mouse ventral mesencephalic cells*	At permissive temperature: Express TH, AADC, NSE, MAP, D2R, DAT, high level of SV40 LT and BDNF At non-permissive temperature: Express TH, AADC, low level of SV40 LT and BDNF and high level of DAT and MAP	Son *et al.* [102]
	ST14A	E14 rat striatum primordial cells	Proliferation at 33°C—express SV40 LT, nestin Differentiation at 39°C—no expression of SV40 LT, express MAP2, NGF, NT3, BDNF, bFGF, CNTF	Cattaneo and Conti [91]
			Differentiation at 39°C—express βIII tubulin, neuron specific enolase, striatal marker DARPP-32	Ehrlich *et al.* [92]
			Express Wnt5a	Peters *et al.* [94]
			Express Wnt2, Wnt5a, Wnt4, Wnt11	Lange *et al.* [95]

Table 2. Continued

Immortalizing agent	Cell line	Cell line derived from	Properties of the cell line	Reference
	Chromaffin cell lines	E17 rat adrenal and neonatal bovine adrenal cells	Proliferation at 33°C—express TH, SV40 LT Differentiation at 39°C—No expression of SV40 LT, express DβH, Phenylethanolamine N-methyltransferase, opoid met-enkephalin, GABA, serotonin	Eaton *et al.* [106, 108]
	RN33B	E13 rat medullary raphe cells	Proliferation at 33°C—express SV40 LT, vimentine, nestin, diffuse neuron-specific enolase, neurofilament Differentiation at 38.5°C—decreased SV40 LT and enhanced neuronal specific protein	Whittemore and White [104]
	RN46A	E13 rat medullary raphe cells	Proliferation at 33°C—express SV40 LT, low level neuron-specific enolase, neurofilament Differentiation at 38.5°C—decreased SV40 LT, express enhanced neuron-specific enolase, low affinity NGF receptor, trk receptor, tryptophan hydroxylase, aromatic aminoacid decarboxylase which are increased with treatment of BDNF, NGF and ACTH and express serotonin	White *et al.* [105]
	H19-7	E17 rat hippocampus	Differentiation at 39°C—express neurofilament, MAP2, no SV40 LT	Eves *et al.* [103]
	3NA12	Postnatal olfactory epithelial cells from H-2K^b-tsA58 (transgenic mice)	At permissive temperature: Express SV40 LT, NCAM, ACIII, OE1, OCNC1, OCNC2 and Gα_{olf} At non-permissive temperature: Express NCAM, ACIII, OE1, OCNC1, OCNC2, Gα_{olf} OMP and no expression of SV40 LT	Barber *et al.* [107]
	PrP$^{-/-}$ ML	Postnatal cerebellar cells from PrP knockout mice	At permissive temperature: positive for nestin, SV40 LT, negative for MAP2, NeuN, GFAP, MBP At non-permissive temperature: reduced SV40 LT expression, positive for MAP2, NeuN and GABA$_A$Rα6, negative for GFAP and MBP	Barenco *et al.* [109]

*This transgenic mouse carry TH-SV40Tag-tsA58 fusion gene. The TH-SV40Tag-tsA58 vector was formed by fusing TH promoter region to the temperature-sensitive mutant form of SV40Tag for tissue specific expression of SV40 Tag. The cells isolated from this transgenic mouse express both TH and SV40 Tag at permissive temperature (33°C) and at non-permissive temperature (37°C); although in the latter the expression of SV40 Tag is less. There was no increase of TH expression at non-permissive temperature, which is contradictory to the expectation. The author also verified that TH expression requires some other factor(s), by co-culturing SN4741 cells with primary mesencephalic cells.

clones have been derived from the transfected cells. The transfected cells were characterized using RT-PCR for the expression of SV40 LT, dopamine transporters (DAT), transcription factor (Nurr1, Pitx3), Wnt1, Wnt5, En1 and TH, which reveals that C2, C3 and C4 express Wnt1, Wnt5a, Nurr1 and En1. Immunocytochemical staining shows that they were immunopositive for nestin. Differentiation by silencing SV40 LT with shRNA-SV40 results in βIII-tubulin and GFAP immunoreactive cells in C1 clone. When differentiated with cAMP/GDNF, C2 and C3 clones exhibit neurite outgrowth in nestin positive cells and later these cells were positive for βIII-tubulin. Transcriptional analysis shows that C2 clone alone expresses Pitx3, DAT and TH. But all the clones fail to express TH at translational level [42].

Immortalization with N-terminal fragment of SV40 LT antigen

AF5 cell lines—The N-terminal fragment of SV40 LT was used in immortalizing rat E14 mesencephalic cells [17, 43]. The vector used in the immortalization was pCMV/SVE/neo (T155/T155g vector), which consisted of a truncated SV40 LT encoding only the N-terminal 155 amino acid (T155). An important feature in the generation of T155g was to preserve the functional p53 in the cellular machinery. This cell line was shown to express T155, neuronal and/or astrocytic markers. The cell line was named AF5. This cell line retained its plasticity and differentiated into βIII-tubulin expressing cells in confluent cultures and 1% of confluent cells were strongly immunopositive for TH [17]. These AF5 cells appear as 'neurospheres' in culture and thought to be similar to neural precursor cells [43]. Upon induced differentiation with serum starvation, this cell line differentiated into GABAergic lineage [44].

Immortalization with SV40 LT antigen conditional vectors

Conditional vectors for immortalization of primary cells could be useful in transplantation studies as well as in clinical therapeutics. The most common SV40 LT conditional vector is the temperature sensitive mutant of SV40 LT.

CSM14.1 cell lines—CSM14.1 cell lines were derived from E14 rat mesencephalic cell, retrovirally immortalized with temperature-sensitive SV40 LT. Undifferentiated cells were positive for neural stem cell marker, nestin at 33°C and upon differentiation at 39°C these cells express neuronal protein MAP5. The cell line also expressed Nurr1 in its undifferentiated state and increased upon differentiation. Further differentiation of these cells led to the time-dependent expression of TH and aldehyde dehydrogenase 2 (AHD2) at translational level [45]. Transplantation of the cells in hemiparkinsonian animals resulted in the reduction of apomorphine-induced rotation [46, 47] and no tumour formation was observed in transplanted grafts or the surrounding host tissue [48].

Factors governing differentiation of neural progenitors into dopaminergic neurons

In the production of dopaminergic cell lines, the cells should express the important factors that govern the cells towards differentiating into dopaminergic neurons, which are briefly described below.

The factors governing dopaminergic phenotype have been extensively reviewed (Fig. 1) [49, 50]. Mesencephalic progenitors that give

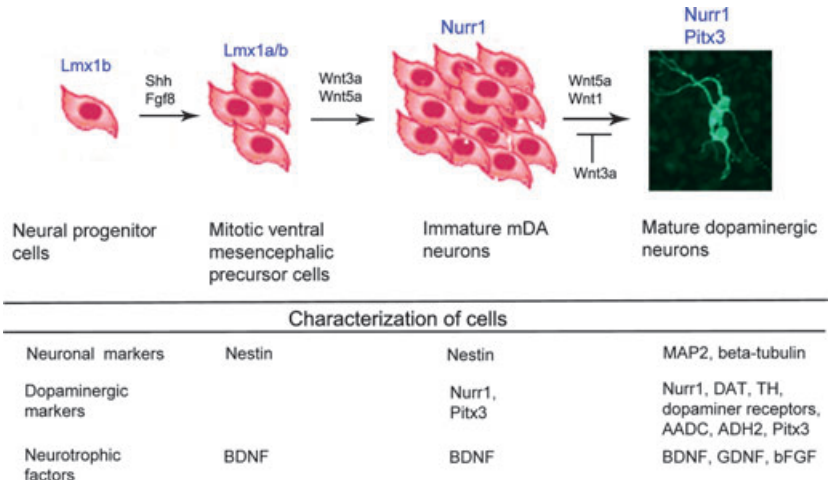

Fig. 1 Overview of development of dopaminergic neurons. Induction of mDA neurons requires shh and FGF8, where shh is required for induction and FGF8 for positioning of mDA neurons. In VM precursors, Wnt1 and Wnt5a (to a lesser extent) induce the proliferation of precursor cells. The up-regulation of Nurr1 positive cells is facilitated by the signalling of Wnt1, Wnt3a and Wnt5a (to a lesser extent). The differentiation is carried out under the influence of Wnt5a in the Nurr1 positive cells. In these differentiating cells, Wnt1 is reduced, Wnt5a is up-regulated and Wnt3a is not expressed as it would inhibit DA differentiation. The cell can be characterized as immature neurons or progenitors by the expression of nestin, mature neurons by expression of β-tubulin and dopaminergic neurons by the expression of DAT and TH. Neurotrophic factor like BDNF, FGF are expressed in immature and mature neurons as they help in neuroprotection and neuroregeneration. Along with other transcription factors and signalling molecules, Lmx1a/b, Nurr1 and Pitx3 promote mesencephalic neuronal progenitors cells towards differentiation into dopaminergic neurons.

rise to dopaminergic neurons are positioned in the isthmic organizer (IsO or known as the midbrain-hindbrain junction) [51]. Isthmic organizer is the centre for organizing the location and size of the mesencephalic dopaminergic (mDA) neurons [52]. The homeodomain transcription factors, Otx2 (from midbrain) and Gbx2 (from hindbrain) are the key factors for the formation of IsO [53]. Sonic hedgehog (Shh) and FGF-8 are the inductive factors for mDA, where Shh is responsible for induction and FGF-8 for positioning of mDA neurons [54, 55]. In rat brain, the maturation of post mitotic mDA neurons takes place between E11 and E15 [56]. The factors influencing mDA genesis and maturation are the transcription factors [57], Pitx3, Nurr1, Lmx1a/b, En1/2 and signalling genes like Wnt.

Factors influencing tyrosine hydroxylase expression

Tyrosine hydroxylase is a rate-limiting enzyme in the production of dopamine and one of the phenotypic markers in mDA neurons. In mouse, TH expression is induced at E11.5 [58, 59]. Along with TH expression, the maturation of mDA neuron is characterized by the expression of DAT at E12-15 [50]. *In situ* hybridization of TH gene expression shows that they are extensively localized in locus coeruleus, substantia nigra (SN) and ventral tegmental area (VTA) [60]. Tyrosine hydroxylase expression is influenced by the following factors.

Lmx1a/b

LIM homeobox transcription factor (Lmx) 1 alpha is considered to be the first transcription factor, which acts upon the mDA progenitor cells that commit for the specification of the cells. In chick embryos, silencing of Lmx1a results in the loss of DA neurons whereas gain of function indicates a robust generation of the mDA neurons [57, 61]. In rats, Lmx1a mutation leads to aberrant brain development [62], with no specific emphasis on mDA development or maturation. Interestingly, Lmx1b was also expressed in the mesencephalon, but their absence did not block the expression TH as studied in Lmx1b null mice. But Lmx1b knockout mice failed to induce Pitx3 and subsequently there was a loss small set of dopaminergic neurons [63].

Nurr1

Nurr1 is a transcription factor belonging to the orphan nuclear receptor family. Nurr1 expression in mDA progenitors starts at E10.5 [64]. Nurr1 is defined as the important component in mDA specification, maturation [65] and it is capable of inducing TH expression [66–68]. Studies on Nurr1 deficient mice show that there is lack of expression of TH, AHD2, D2R [69], VMAT2 and DAT [65]. In Nurr1 mutant, engrailed 1 and 2, AHD2, AADC has been reported to be reduced or absent by E15.5 [65, 70]. Nr4a2—null mice (Nr4a2 also known as Nurr1) died soon after birth, which revealed that these embryos have no TH expression. Nurr1 is also responsible for the expression of VMAT [65, 69, 71, 72]. From knockout studies, it becomes obvious that Nurr1 is necessary for the expression of many genes involved in the DA system primarily TH, AADC, VMAT and DAT.

Pitx3

Pitx3 is a bicoid-related homeobox protein, expressed prominently in mDA neurons [73]. *Aphakia* mice, a recessive phenotype with a double genomic deletion in Pitx3 gene [74], express TH-positive neurons in SN till E11.5 and become scarce after E12 [67, 75–77]. Studies on Pitx3 knockout mice prove the same [78]. Pitx3 is known to activate TH promoter *via* a high affinity-binding site, which appears to be cell dependent [79]. Immunohistochemical analysis in mice brain shows that, Pitx3 and Nurr1 cooperate with each other in the regulation of TH gene expression [80]. In Pitx3 deficient mice there is lose of TH expression in SN, but not in VTA [78]. Pitx3 and TH expression is completely overlapped throughout SN and VTA [76], suggesting that TH is indeed in the control of Nurr1 and not under Pitx3 [65]. Research indicates the involvement of microRNA-133b in the regulation of Pitx3 *via* negative feedback regulation. It is found that miR133b is deficient in the midbrain of PD patients [81]. But the recent report [82] has stated that genetic predisposition of Pitx3 and miR-133b did not contribute to the risk of PD. The reason for the miR-133b deficiency in the midbrain of PD patients might be because of the feedback mechanism.

Wnt family

The other factors influencing TH expression are the members of the Wnt family [83]. The Wnt are a family of glycoprotein that regulates cell proliferation, cell fate decision and differentiation. The commonly studied Wnt in dopamine neurogenesis are Wnt1, Wnt3 and Wnt5a [84]. Loss of function studies revealed that Wnt1 deficient mice were not able to develop any midbrain or anterior hindbrain structures, showing that Wnt1 is necessary for midbrain development. In rats, Wnt1 is highly expressed in ventral midbrain at E11.5 [85, 86]. Wnt1 induces Fgf8 expression [87]. In E14.5 primary ventral midbrain cultures, on addition of exogenous Wnt5a increases TH-expressing neurons and also up-regulates Pitx3 and cRET mRNA [83]. It is understood that Wnt1 acts as a mitogen to the neuronal progenitors, whereas Wnt5a acts as a differentiating agent by inducing TH expression in $Nurr1^{+}$ cells.

Characteristic features of SV40 LT antigen immortalized cell lines

The SV40 LT immortalized cell lines derived from mesencephalic progenitors exhibit either neuronal and/or astrocytic properties (Fig. 2). But most immortalized cell lines like AF5, $1RB_3AN_{27}$, CSM14.1 and iVMP were found to be neuronal progenitors. This may be because of the commitment/predetermination of the cells and the cellular niche from which they have been isolated. When differentiated, some of these cell lines behave as dopaminergic neurons upon transplantation or favourable conditions of differentiation. The expression of dopaminergic factors in the SV40 immortalized cell lines are described next.

Lmx1a/b expression

No study has been performed in the immortalized mesencephalic cells, which are described above with the exception of iVMP cells.

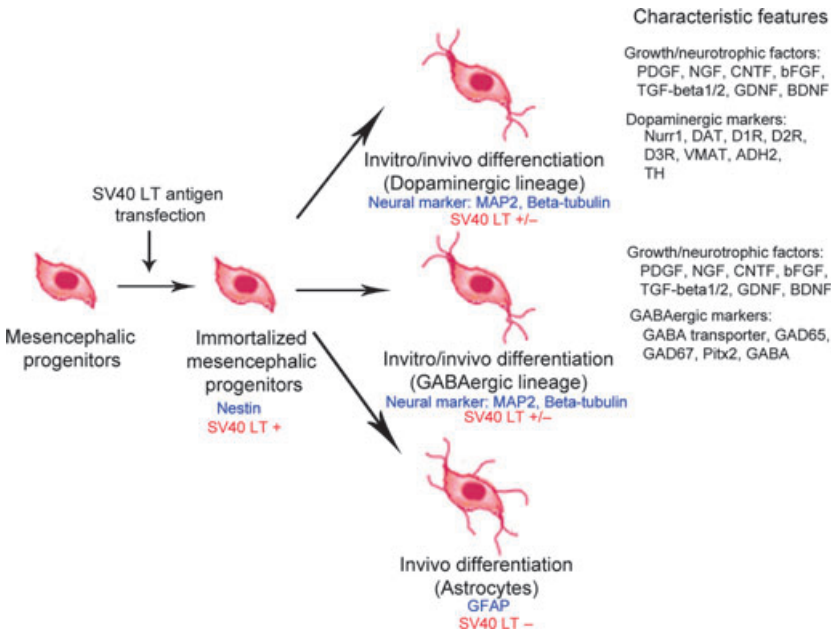

Fig. 2 Overview of gene expression in immortalized cell line with SV40 large T antigen. The transfection of mesencephalic progenitors with SV40 large T antigen leads to the production of immortalized progenitors cells. These neuronal progenitors express nestin, a neuronal stem cell marker and they express the large T antigen. Upon differentiation *in vivo/in vitro*, the immortalized neuronal progenitors differentiate into dopaminergic or GABAergic or astrocytic phenotype. The dopaminergic differentiation is characterized with the expression of β-tubulin, TH, DAT, dopamine receptors D1R, D2R, D3R and VMAT. The GABAergic differentiation is characterized with the expression of β-tubulin, GABA and Pitx2. The dopaminergic and GABAergic differentiated cells may also express neurotrophic factors like BDNF, GDNF and bFGF. In some cases, upon transplantation these immortalized neuronal progenitors differentiate into astrocytes with the expression GFAP and GDNF. The expression of SV40 large T antigen in these differentiated cells is dependent on the vector design (conditional vector) or the *in vivo* conditions.

Recent experiments on embryonic stem cells, show that forced expression of Lmx1a has increased the production of dopaminergic neurons [88]. Lmx1b expression has been observed in iVMP clones. During differentiation, some of the iVMP clones have an increased expression of Pitx3 [42], reminding that Lmb1b controls Pitx3 expression [63].

Pitx3 expression

Pitx3 expression has not been reported in the cell lines AF5, $1RB_3AN_{27}$ and CSM14.1. C2-iVMP clone exhibits Pitx3 expression after differentiation with cAMP/GDNF and rest of the clones (C1, C3 and C4) did not express Pitx3 [42]. The expression and the molecular mechanism of Pitx3 in the cell lines need to be further examined. The failure of Pitx3 expression in other clones might be tied with the miRNA regulation of Pitx3 or SV40 LT might interfere with the Pitx3 regulation.

Pitx2 expression

Interestingly, AF5 cell lines express Pitx2; a transcription factor belonging to the same homeodomain transcription factor as Pitx3, which is involved in neuronal differentiation. Pitx2 expression was observed in the developing diencephalon, mesencephalon and ventral spinal cord [89], which is suggestive of its contribution in the development of GABAergic neurons. Pitx2 and GABA were also detected in post mitotic ventral mesencephalic neurons [90]. The AF5 cell lines express Pitx2 along with GABA and differentiate into GABAergic lineage [44]. These cell lines were isolated on E14 from mesencephalic cells, but they differentiate into GABAergic neurons (not as dopaminergic neurons). We have to take into account that the cell lines were strongly immunopositive for TH [16]. This shows that mesencephalic cells could be differentiated into dopaminergic or GABAergic phenotype. The other conclusion can be obtained from the AF5 cell line is that it might have been immortalized from mixed cell population (both GABAergic and dopaminergic precursors), leading to the different outcome in this cell line [43, 44], indicating that GABAergic precursors might have prevailed over the dopaminergic precursor cells (see Table 2).

Nurr1 expression

The cell lines AF5 and $1RB_3AN_{27}$ are not analysed for the expression of transcription factor, Nurr1. These cell lines might express Nurr1, as they were derived from E14 rat primary mesencephalic cells, which usually develop into mDA neurons and express TH. Therefore, Nurr1 expression should be investigated.

In CSM14.1 cell line, Nurr1 is expressed both in permissive and non-permissive temperature. In non-permissive temperature, there is

an increased Nurr1 expression that in turn drives the expression of TH and AHD2.

The iVMP E12 neuronal progenitor cell line expresses Nurr1, which is promising to develop dopaminergic cell lines *in vivo*. It demands further research to demonstrate the *in vivo* dopaminergic differentiation of iVMP E12 neuronal progenitor cell lines.

Wnts expression

Expression of Wnts in the cell lines AF5, $1RB_3AN_{27}$ and CSM14.1 has so far not been studied. The clonal cells (C2, C3 and C4) express Wnt5 *in vitro*. But these clones do not exhibit TH-protein expression [42]. Obviously, SV40 LT might hinder TH expression and no other explanation can be made, if Wnt5 is indeed a differentiating agent in mesencephalic cells.

Expression of Wnts has been studied in ST14A cell lines, which are established by immortalizing E14 rat striatum primordial cells by temperature-sensitive mutant of SV40 LT. These cell lines proliferate at 33°C with the expression of nestin and differentiate at 39°C with the expression of MAP2. These cells express SV40 LT at 33°C, but not at 39°C [91]. Upon differentiation, these cells express β-tubulin, striatal marker DARPP-32 and reported to have the property of medium-sized spiny neuron [92].

The ST14A and its derivatives CNTF-ST14A and GDNF-ST14A were found to express Wnt5a. After the temperature shift from 33 to 39°C, there is an increase in the expression of Wnt5a [93–95]. Overexpression of CNTF and GDNF in ST14A cell lines results in overcoming stress response during the early stage of differentiation *via* Ras/MAP kinase pathway, which might be a suitable candidate to treat neurodegenerative disease like HD [96] and expression of genes regulating cell migration and differentiation of neuronal progenitors [97] respectively. Wnt5a is reported to be associated with the increase of TH expression in dopaminergic neurons [83]. Nevertheless it may be possible that this cell line might differentiate into TH-expressing neurons, as it expresses Wnt5a, which needs to be evaluated by future studies. Transplantation of ST14A cells along with glioma N29 cells into the caudate nucleus shows that these cell lines can inhibit glioma outgrowth *in vivo* [98]. So, this can be deployed beneficially in cell therapeutics.

TH expression

The cell lines AF5, $1RB_3AN_{27}$ and CSM14.1 were derived from mesencephalic progenitors and found to be positive for the expression of TH, which indicates that these cells are committed towards dopaminergic neurons. Interestingly, AF5 cell line is found to be GABAergic lineage rather than dopaminergic. The absence of TH expression might be because of the phenomenon of time-dependent expression as described by Chung *et al.* [99]. Genetic manipulation of primary fibroblast to express TH failed in a long-term fashion [100, 101]. When SN4741 cell line co-cultured with mesencephalic neurons and astrocytes, TH expression seems to be greatly increased in the SN4741 cell line indicating that TH expression is regulated by distinctive factors [102]. To analyse the expression of TH, further research is required to clarify the intrinsic or/and extrinsic factor necessary for the expression of TH *in vivo* and those that are absent in certain *in vitro* conditions.

SV40 LT antigen expression *in vitro* in immortalized neuronal cell lines

All the cell lines immortalized with SV40 LT express the antigen. The cell lines (ST14A, chromaffin cell lines, RN33B and H19-7) established with temperature-sensitive SV40 LT vector expresses LT at 33°C and not upon temperature shift to 39°C in which they were able to differentiate [92, 103–109]. Refer transplantation studies for *in vivo* expression of SV40 LT by the immortalized cell lines.

Interaction of SV40 large antigen in differentiation

Most of the immortalized cell lines with LT lose their property to differentiate into specialized cell types with few exceptions. It is suggested that the binding and inactivation of cellular proteins such as p53, p300, p107, p130 and Rb by LT leads to de-differentiation or inability to differentiate in the presence of LT [110]. LT was also reported to inhibit myogenic differentiation in mouse skeletal muscle cell line by suppressing the expression of myoD gene family, partially through inducing c-jun [111]. Dis-immortalization with cre/lox or conditionally immortalized LT cell line may overcome this problem. Temperature sensitive LT immortalized cell lines like ST14A were able to differentiate into glial and neuronal cells at non-permissive temperature of 39°C [91, 92], which apparently proves the fact that LT inhibits differentiation in cells.

The iVMP E12 neuronal progenitor cell line reveals that LT might interfere with gene expression related to dopaminergic factors. These cell lines were found to express all the markers including TH mRNA (Table 2). From this it is evident that these cell lines were able to produce TH mRNA, but there was no protein expression. The authors would like to hypothesize that LT interferes with dopaminergic differentiation probably by directly binding to post-transcription or/and translation machinery.

Transplantation studies

In PD's animal models with SV40 LT antigen immortalized cell lines

The transplantation of foetal mesencephalic tissue into the striata of 6-OHDA lesion rats [3, 4] and hemiparkinsonian rats [112] shows that there is improvement in drug-induced rotations. In PD's models, few studies have addressed the transplantation of SV40 immortalized cell lines (Table 3).

Drug-induced rotation test

The 6-OHDA lesion rats transplanted with $1RB_3AN_{27}$ cell lines are found to have reduction in methamphetamine-induced turning with an improvement of neurological deficits [40, 113, 114]. Likewise, conditionally immortalized cells like CSM14.1 have also been shown to reduce apomorphine-induced rotation in hemiparkinsonian animals [48]. Palmer *et al.* [115] reported that there is a reduction in drug-induced rotation in 6-OHDA treated rats, transplanted intra-nigrally

Table 3 Transplantation studies with immortalized cell lines

Cell line	Traplantation model	Duration of post-trans plantation experiments	Chracteristic features *in vivo*	Drug induced rotation	Reference
CSM14.1	Hemiparkinsonian animal	Apomorphine-induced rotation: after 3, 6, 9 and 12 weeks Histology: 12 weeks	Express GFAP, NeuN No tumour formation, no SV40 LT and TH expression	Reduce apomorphine-induced rotation	Hass *et al.* [48]
$1RB_3AN_{27}$	6-OHDA lesion rats	Methamphetamine induced rotation: after 30 days Histology: after 30 days	No SV40 LT expression, no tumour formation	Reduced methamphetamine induced turning with an improvement of neurological deficits	Adams *et al.* [40]
iVMP	6-OHDA lesion rats	Histology: after 7 and 14 days	Express SV40 LT upto 7 days Express nestin, GFAP and beta-tubulin positive cells in the graft No TH-positive cells were observed		Nobre *et al.* [42]

with dopaminergic neurons. Likewise, the reduction of drug-induced rotation in transplanted PD's animal model using cell lines might be because of the *in vivo* differentiation of the cell lines into dopaminergic neurons.

Transplantation of $1RB_3AN_{27}$ cell lines in normal and 6-OHDA lesion Sprague-Dawley rats shows that these cell lines did not form tumours in transplant grafts. It was found that these cell lines did not divide or produce LT. Furthermore, they did not elicit immune response or extend the neuritis and were not rejected by the host. It was suggested that there was an inhibiting factor in brain that inhibits LT. *In vitro* experimentation with the soluble fraction from brain inhibits SV40 LT expression in $1RB_3AN_{27}$ as well as growth [40, 113, 114, 116, 117]. Likewise, immortalized cell lines of olfactory ensheathing glia with SV40 LT upon transplantation in animal model of spinal cord injury shows that there was no LT expression in the grafts after 4 weeks of transplantation and the animals' recovered sensory and motor function [118]. CSM14.1 cell line on transplantation in hemiparkinsonian rats did not form tumours and were not able to express SV40 LT [48]. In conditionally immortalized chromaffin cell line with SV40 LT derived from E17 rat adrenal and neonatal bovine, adrenal cells were not exhibiting LT expression when transplanted in the lumbar subarachnoid space of spinal cord [106, 108]. Chromaffin cells have also been used in the cell therapy treatment of PD, as these cell line express TH and dopamine β hydroxylase, but these cells survive poorly after transplantation to the striatum [6, 119, 120]. To avoid further consequence of tumour development by SV40 LT, these cell lines can be dis-immortalized with Cre/lox site directed recombination [108, 121, 122]. Thus, the SV40 LT immortalized cell lines might be transplanted into the host without any implication of forming tumours.

The summary of the transplantation studies in animal models with SV40 LT immortalized cell lines results in the following: (1) the cells do not proliferate in the grafts, (2) they were not rejected by the host, (3) they did not form tumours, (4) they did not express SV40 LT in the grafts and most importantly (5) helps in recovering drug-induced rotation, a test to check functional aspect of the integrated grafts in PD's animal models.

In PD's animal models with stem cells

Stem cells have attracted particular attention in recent years, because of their potential to differentiate into desired cell types. They are promising in transplantation for non-curable neurodegenerative disorders. Human stem cells used for dopaminergic differentiation are of different origins namely, mesenchymal stem cells [123], neural stem cells [124–126], amniotic fluid stem cells [127] and embryonic origin [128].

Human neural stem cell (hNSC) lines

In vitro experiments with (1) HB1.F3 (hNSCs supernatant on human derived dopaminergic SH-SY5Y cells) show that they prevent apoptosis induced by 6-hydroxydopamine [126] and (2) ReNCell VM (hNSCs isolated from developing mesencephalon) differentiate into dopaminergic neurons, upon 'preaggregation differentiation' [124]. Thus, *in vitro* experiments reveal that these cells can differentiate into dopaminergic neurons as well as provide neuroprotection. Transplantation studies with hNSCs shows that they survive, migrate and differentiates into astrocytes in ischemic rats [125]. When transplanted in Primate Parkinson's model the cells differentiates into TH and DAT positive neurons [129], and in 6-OHDA rats they exhibit MAP2, but rarely express TH [126]. When hNSCs transplanted in the spinal cord, the cells differentiate into astrocytes and GABAergic neurons [130]. In case of human amniotic fluid stem cells, they do not generate dopaminergic neurons *in vitro* or after transplantation *in vivo* [127].

In conclusion, hNSCs upon transplantation provides neuroprotection, differentiates into astrocytes, dopaminergic/GABAergic neurons.

Even if hNSCs differentiates into TH-positive neurons, they survive poorly and were less TH-positive cells in the transplanted grafts.

Human embryonic stem cell (hESC) lines

In context to transplantation in PD, hESCs have been given more importance. Human embryonic stem cells are derived from the inner mass of human blastocyst. The established hESCs used in the successful production of dopaminergic neurons *in vitro* are H1, H9, HES-1, BGO, MBO3, HSF-6, SNU-hES3, khES-1, HUE-1, SNUhES1 and SNUhES16. Most of the stem cells develop into dopaminergic neurons *in vitro*, with the expression of TH [128, 131–144]. Co-culturing of hESCs with feeder cells such as stromal cell lines [142] and midbrain astrocytes [138], increase the production of TH-positive neurons. The differentiation mechanism behind astrocytes is still to be identified; nevertheless it is hypothesized that differentiation might be as a result of the action of the growth factors such as BDNF and GDNF produced by astrocytes. But in case of stromal cells, apart from secreted factors like FGF, hepatocyte growth factor and VEGF, IFG2 and pleiotrophin (PTN) were observed in high level, which shows that these factors (IFG2, PTN) are involved in successful differentiation of hESCs into dopaminergic neurons [145].

Transplantation with either undifferentiated hESCs or differentiated hESCs towards dopaminergic neurons survives in the graft, but less TH-positive cells were observed in the transplanted grafts [132–142]. It shows that although differentiation of hESCs into neuronal/dopaminergic neurons is successful *in vitro*, whereas it fails *in vivo*. Although these cells do possess all the characteristic features of dopaminergic neurons, they were not able to efficiently differentiate *in vivo*. This might be because of the transplanted cell niche and the surrounding factors that inhibit production of TH-positive neurons and interfere with survival.

The most important disadvantages in the transplantation of both hESC and hNSC lines are (1) these cells evoke immune response in host after transplantation (because of heterologous transplantation nature) and (2) undifferentiated cells or stem cells cultured along with feeder cells will result in the formation of teratoma/tumour. Berderlau *et al.* [133] have shown that pre-differentiated hES cells exhibit tumour formation in transplanted 6-OHDA lesion rat model of PD. Likewise, transplantation with foetal NSC in a boy with ataxia telangiectasia results in multifocal brain tumour, which is of donor origin [146].

Human mesenchymal cells

Mesenchymal stem cells (MSC) derived from bone marrow will be a better choice to avoid the above-mentioned disadvantages in treating PD. Because of the autologous transplantation nature of MSC, these cells will not exhibit neither teratoma/tumour nor elicit immune response.

Mesenchymal stem cells can be differentiated towards dopaminergic neurons with different combinations of BDNF, GDNF, neurturin, neurotrophin 3, FGF-8, TGF-β, SHH, oestrogen and retinoic acid [147]. Transplantation of hMSCs helps in reducing the decline of TH-positive cells, thereby differentiating into TH-positive neurons [148], and therefore results in improvement of behavioural defects and exhibit dopaminergic phenotype [149]. In LPS-induced *in vivo* and *in vitro* models, treatment with hMSCs is known to decrease microglial activation, TNF-α, iNOS and also reduce the production of NO. *In vitro* experiments with co-culture of microglia and mesencephalic neuron along with hMSCs found that hMSCs reduce the loss of TH-positive cells [150]. Autologous hMSCs delivered intra-arterial and intravenously in multiple system atrophy (MSA) patients proved to be beneficial by delaying the progression of neurological defects, without adverse effects [151].

In general, hMSCs proves to be significant in recovering neurological defects (PD, MSA) by the following mechanism(s): (1) neuroprotection, (2) differentiation into dopaminergic neurons, (3) delaying the progression of neuronal damage and (4) prevention in the decline of TH-positive cells. An important advantage is that these cells can be injected intra-arterially and intravenously, without serious consequences.

Cell/tissue transplantation in PD patients

Several open-label trials with foetal mesencephalic tissue or cells in PD patients have been carried out since 1987 (Table 4) [152, 153]. Later double-blind trials were carried out, which were not promising [9, 154, 155]. The patients who received the grafts were reported to have significant improvement in motor function [10, 156–162] and have been maintained for more than 10 years in some patients [152, 153, 163–165]. The grafted neurons were also found to integrate and release dopamine [10]. Post-mortem reports of PD patients who died of unknown causes have been reported to have large number of dopaminergic neurons in the transplanted grafts after 3–4 years of surgery [9, 163–167].

Few transplantation studies in PD patients were given TH expression parameters (Table 4). It is not ethical to get biopsy samples for assay for TH expression. Non-invasive techniques like PET, MRI help to overcome this barrier, PET studies with flurodopa uptake in PD transplanted patients shows that there is an increase in flurodopa uptake, which signifies the functional aspect of the transplanted grafts [156, 159, 160, 166, 168–171]. Autopsy samples from transplanted patients who died of other causes have shown that the graft contains TH immunoreactive cells [159, 160, 166, 172].

However, recent reports on these transplantation studies, illustrate that the grafts contain α-synuclein-positive Lewy bodies. But these grafts are also reported to have dopaminergic neurons with the expression of tyrosine hydroxylase [163, 164]. Mendez *et al.* [165] reported that there was no such PD pathology observed in the post-mortem of transplanted PD patients. These studies in transplantation of foetal mesencephalic tissue or cells in PD patients pose a new dimension in the study of PD pathology. These studies raise several questions on cell transplantation in PD. The positive aspect of cell transplantation in PD is that the transplantation helps PD patients with a long-term symptomatic relief to certain extent, but not with PD pathology *i.e.* α-synuclein-positive Lewy bodies in grafted/transplanted cells. The clinical studies will be of great help in learning more about PD pathology.

The report of Mendez *et al.* [165] showed that no such PD pathology was observed in transplanted grafts. This may be hypothesized

Table 4 Transplantation studies in PD patients with cells/tissues

Cell type/tissue	Site of transplantation	Outcome	TH expression	Remarks	Reference
Foetal mesencephalic cells/tissue	Caudate nucleus	Clinical recovery persisted in 7 of the 10 patients 5 years after implantation	ND	Improved motor function	Lopez-Lozano *et al.* [172]
	Putamen and caudate nucleus	Free of complications for 1 year after transplantation	ND	Modest improvement	Markham *et al.* [182]
	Bilateral post-commissural putamen	Increased flurodopa uptake	Autopsy results shows grafts were viable, integrated into the host striatum with dense dopaminergic neurons positive for TH immunoreactivity.	Improvement in motor function	Kordower *et al.* [160]
	Putamen	Increased [18F]dopa uptake suggesting the graft survival	ND	Significant and sustained improvement in motor function	Lindvall *et al.* [169]; Sawle *et al.* [168]
	Putamen	Bilateral improvement of motor functions	ND		Leviver *et al.* [183]
	Straitum and substantia nigra	Increased [18F]dopa uptake suggesting the graft survival	Exhibit dopaminergic phenotype by expressing TH and provide nerve innervations.	No motor complication observed	Mendez *et al.* [166]
	Intra-striatum	Increased [18F]dopa uptake suggesting the graft survival	ND	Modest improvement	Peschanski *et al.* [170]
	Caudate nucleus	Bilateral motor improvement [18F] fluorodopa before and after surgery revealed bilateral restoration of caudate dopamine synthesis in a patient	ND	Modest improvement	Spencer *et al.* [171]
	Bilateral caudate and putamen	Bilateral motor improvement [18F] fluorodopa before and after surgery revealed uptake in both putamen and caudate nucleus	ND	Modest improvement	Brundin *et al.* [156]
	Unilateral striatum later into putamen or both putamen and caudate nucleus	Sequential transplantation does not interfere with either first or the second transplantation	ND	Modest improvement	Hagell *et al.* [158]

Table 4. Continued

Cell type/tissue	Site of transplantation	Outcome	TH expression	Remarks	Reference
Foetal mesencephalic c ells/tissue (Porcine)		No adverse effects Improved gait in some patients	ND	Modest improvement	Schumacher *et al.* [184]
Adrenal medulla (autograft)	Caudate nucleus	Complications were observed in some patients; but shows improvement	ND	Improvement in Parkinson's symptomatology	Lopez-Lozano *et al.* [185]
	Caudate nucleus	Distinct and persistent improvement seen in some of the younger patients	ND		Allen *et al.* [186]
Co-transplantation of Adrenal medulla and foetal ventral mesencephalon	Caudate nucleus	Clinical recovery lasted in a step-wise manner for 3 years after transplantation	Autopsy report demonstrates that large number of TH-positive cells survives after 1 year after implantation	Long-term improvement in seriously disabled Parkinson's patients	Lopez-Lozano *et al.* [187]
Foetal substantia nigra and adrenal medulla	Caudate nucleus	Improvement in young patients, but a high morbidity and mortality rate in elderly patients	ND	Modest improvement	Madrazo *et al.* [188]
Foetal nigral cells	Bilateral Post-commissural putamen	Increased putamenal flurodopa uptake suggesting the transplantation is helping n improvement	Autopsy report from two patients shows that there is robust survival of TH immunoreactive cells and abundant reinnervation of the post-commissural putamen	Long-term improvement appears to be in consistent with the transplantation	Hauser *et al.* [159]
Bone marrow derived mesenchymal stem cells (autologous)	Unilateral sublateral ventricular zone	Improvement in overall well-being, facial expression, gait and reduction in freezing episodes	ND	Modest improvement	Venkataramana *et al.* [189]

ND, not described.

by the following reasons: (1) the grafts might contain both dopamine and serotonin neurons (a mixed cell population), so they might act as an inhibitor or as a buffer to reject Lewy body expression or integration upon migration and (2) the grafts may not be triggered by the factor(s) necessary to stimulate the expression of Lewy body.

The questions which can be asked is whether the α-synuclein-positive Lewy bodies are integrated into the grafts through migration from the host-to-graft or is the graft been triggered by external factors to express α-synuclein-positive Lewy bodies. Follow-on studies should address this issue to have a better understanding in transplantation in PD.

Conclusion

The SV40 immortalized neuronal cell lines are promising in elucidating the interaction of SV40 LT with other necessary factors for the generation of dopaminergic neurons. In *in vitro* studies, immortalized cells can be used as feeder cells in culturing primary VMP cells to enhance the production of dopaminergic neurons and in stem cells towards differentiating into dopaminergic phenotype, owing to the expression of trophic factors. The immortalized cell line with LT upon transplantation in animal models of PD shows that they are able to reduce drug-induced rotation, without any implication of tumour formation. Still open questions exist to be answered in the use of these cell lines. Certain cell lines were not able to express TH *in vitro*, but managed to express *in vivo* (indirect evidence like dopamine production, reduction of drug-induced rotation). Hence, the future studies should address the extrinsic and/or intrinsic factor triggering TH expression. It will also very interesting and useful to address the interaction of SV40 LT with any proteins involved in dopamine synthesis. Most of the cell transplantation experiments have not shown any pathological facets as revealed in PD patients. Further research is needed to study whether or not PD pathology is observed in transplantation studies in animal models. Although, SV40 LT immortalized neuronal cells favour recovery without tumour formation in PD animal models, they are not suitable to administer in patients with PD because of ethical issues. In future, human stem cells may offer a promising hope for cell-based transplant treatment of neurodegenerative diseases, owing to their unique properties in differentiating into dopaminergic neurons and in delaying the progression of neurological defects without adverse effects.

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Conflict of interest

The authors declare that there is no conflict of interest.

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