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Application of Embryonic Stem Cells in Parkinson's Disease

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1. Introduction

The nervous system is a stimulating target for regenerative medicine. Parkinson's disease (PD), which afflicts over a 1 million people in the US, is a chronic neurodegenerative disorder characterized by the degeneration and death of midbrain neurons that produce the neurotransmitter dopamine (DA), resulting in tremors at rest, an inability to initiate or complete routine movements, muscle rigidity, postural instability, and lack of facial expression. Although the etiology of idiopathic PD is not known, several predisposing factors for the dopaminergic depletion associated with the disease have been suggested, including programmed cell death, viral infection, and environmental toxins.
DA neurons, in the substantia nigra pars compacta, play a prominent role in the control of many brain functions, such as voluntary movements and many behavioral processes (Maxwell & Li, 2005). These neurons can be identified via the expression of some specific transcription factors, including Engrailed 1 (EN1), PITX3, NURR1, and LMX1b, which are also very important in the development of DA neurons (Smidt et al., 2003).

2. Current therapeutic strategies for PD

2.1 Drug therapy and DBS

Current established therapeutic strategies for PD patients comprise drug treatments such as L-dopa (a precursor of dopamine), DA agonists, enzyme inhibitors and deep brain stimulation in the thalamus, subthalamic nucleus and globus pallidus (Figure 1). However, these treatments are effective in early stage and can temporarily ameliorate symptoms and cannot cure the disease. Therefore, there is a need for novel therapeutic approaches which one of them is to regenerate the damaged tissue. Since direct regeneration of brain tissues is difficult to achieve, an alternate supply of neural cells is required in order to attain any therapeutic goal. Cell replacement therapy (neurotransplantation) has been suggested to have a great potential for restorative therapy in PD (Freed et al., 2001; Hagell & Brundin, 2001; Olanow et al., 2003).

2.2 Cell replacement therapy

2.2.1 Foetal mesencephalic tissue

Transplantation of human foetal ventral mesencephalic tissues into the putamen or caudate nucleus of PD patients has been adopted as a potentially curative cell replacement therapy

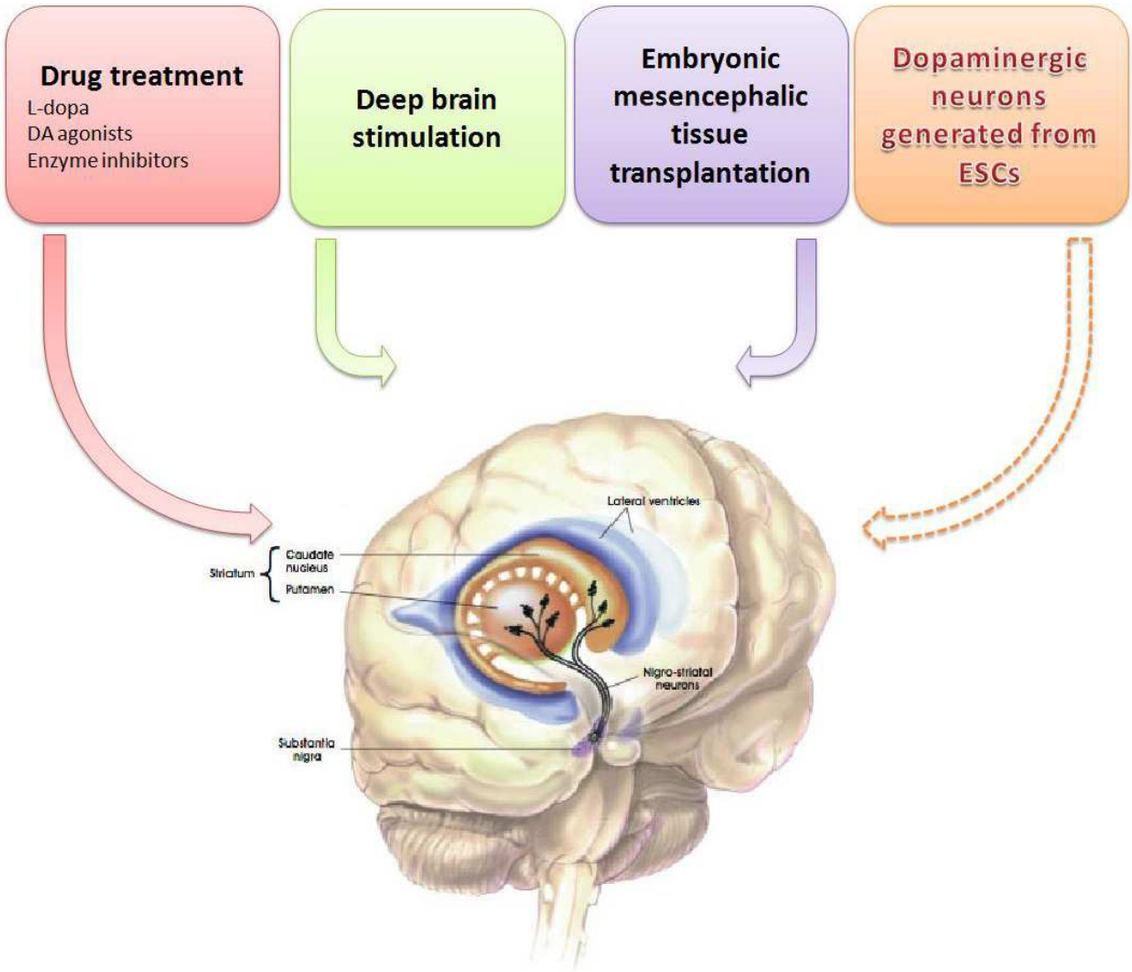

Fig. 1. In the normal brain, DA neurons located in the substantia nigra send their axons to the striatum (i.e. the putamen and caudate nucleus). In the PD brain, the main pathology leading to motor symptoms is a degeneration of these neurons causing a loss of DA in the striatum. Drug treatment, deep brain stimulation and embryonic mesencephalic tissue transplantation are current therapeutic approaches for PD. Transplantation of DA neurons generated from embryonic stem cells has been suggested to restore striatal dopaminergic innervation thereby alleviating PD symptoms.

with long term survival of grafted cells (Kordower et al., 2008a; Li et al., 2008; Mendez et al., 2008). A bulk of experimental and clinical studies have shown functional efficacy of grafting of embryonic mesencephalic tissue into the striatum and a biological mechanism underlying the observed improvement. In spite of promising results of foetal mesencephalic cells transplantation, so far numerous unresolved problems remain to be addressed, such as ethical and religious questions and logistics of acquiring foetal tissues, graft-induced off-medication dyskinesias in up to 56% of transplanted patients (Freed et al., 2001; Hagell et al., 2002; Olanow et al., 2003) and inadequate foetal tissues for transplantation (since treatment of a single PD patient requires DA neurons from six to ten human aborted foetuses). To bypass these difficulties, neurons with a DA phenotype generated from embryonic stem cells (ESCs) could be employed as a practical and effective alternative for foetal brain tissues for transplantation.

2.2.2 Embryonic stem cells

ESCs, derived from the inner cell mass of early post-fertilization blastocysts, are capable of unlimited cell expansion *in vitro* while maintaining their pluripotency. These characteristics of ESCs make them an excellent source of functional differentiated cells for cell replacement therapy of neurodegenerative medicine such as PD, provided that reliable means of inducing differentiation to specific cell types can be achieved. After differentiation, ESCs-derived neurons have to work at least similar to those in embryonic mesencephalic transplantations. Therefore, these neurons have to achieve the following requirements to improve PD markedly after grafting: (i) release DA and exhibit the molecular, morphological and electrophysiological properties of midbrain DA neurons (Mendez et al., 2005; ,Isacson et al., 2003); (ii) reverse motor deficits in animal models resembling the symptoms in patients; (iii) enable 100000 or more grafted DA neurons to survive long term in each human putamen (Hagell & Brundin, 2001); (iv) re-establish a dense terminal network throughout the striatum; and (v) become functionally integrated into host neural circuitries (Piccini et al., 2000).

Two basic strategies can be employed to use ESCs as a cell source for cell replacement therapy: they can be used without any previous in vitro differentiation based on the hypothesis that regional microenvironment is the best inductive cue to obtain the required cell type. An alternative strategy would be to partly or totally differentiate ESCs into the desired cells based on the hypothesis that the host tissue-derived inductive cues are not sufficient to achieve this and to avoid tumor formation at the same time.

Although it has been reported that transplantation of undifferentiated ESCs into the midbrain parkinsonian rats resulted in dopaminergic differentiation of these cells, high rate of differentiated cells were serotonergic neurons (a relation of 2:1 of dopaminergic to serotonergic neurons). Due to this fact that serotonergic neurons in grafts are responsible for off-medication dyskinesias in clinical transplantation studies (Carlsson et al., 2007), it is rather unlikely that the resulting cell composition is well suited for successful transplantation. A more limiting issue was that 20% of the grafted animals had to be sacrificed before the defined study endpoint because of teratoma formation. Although several strategies have been employed to reduce the risk of tumor formation (Chung et al., 2006; Li et al., 1998; Schuldiner et al., 2003), the use of undifferentiated ESCs remains an unsafe strategy.

3. In vitro neural differentiation of ESCs

The in vitro differentiation of ESCs toward dopaminergic neurons has followed different culture protocols in the presence of various combinations of growth factors and signaling molecules. By studying these signaling molecules present in the midbrain microenvironment during development and in the adult, and which key regulatory transcription factors the cells express, protocols for controlling *ex vivo* dopaminergic neurons differentiation can be achieved.

3.1 The major signaling molecules

Retinoic acid (RA) is one of the most important signaling molecules that promote neuralization in embryos and later in development (Bain et al., 1996; Guan et al., 2001; Diez del Corral & Storey, 2004). All-trans RA, which can bind to both RAR subtypes, is commonly employed to induce neuronal differentiation *in vitro*. RA induces a pan-neuronal differentiation and the cell

population obtained after application of this differentiation factor is relatively heterogeneous (Carpenter et al., 2001, Schuldiner et al., 2000). Takahashi *et al* showed that cells cultured with RA and fetal bovine serum (FBS) expressed markers for GABAergic, dopaminergic, and cholinergic neuronal phenotypes at low levels (Takahashi et al., 1999).

Wnt3a is another signaling molecule recently shown to play a key role in regulating neurogenesis in the adult brain. *In vivo* expression of a Wnt3a inhibitor reduced neurogenesis in the adult hippocampus. By contrast, Wnt3 overexpression *in vivo* and *in vitro* increased neuronal differentiation (Lie et al., 2005). However, Wnt3's overexpression *in vitro* resulted in a mixed culture of glia and neurons. Also, it is yet to be determined whether Wnt signaling induces a specific neuronal phenotype or serves as a nonspecific, pan-neuronal signal.

BMP is another inhibitory signal found within neural differentiation. It has been shown that the overexpression of the BMP antagonist Noggin increased neural differentiation in neurosphere culture (Setoguchi et al., 2004). BMP signaling activation in undifferentiated cells can result in extraembryonic endoderm committed cells and epidermogenesis. In addition, it has been reported that BMP4 can induce mesodermal differentiation. Noggin is a well characterized BMP2 and BMP4 antagonist and has been shown as neural inducer in *Xenopus* embryos (Niknejad et al., 2010). Since ESCs are pluripotent and susceptible to give rise to all three germ layers, blocking BMP signaling using its antagonist noggin can induce neuronal differentiation by its inhibitory effects on the mesodermal, endodermal, and epidermal fate of ESCs (Gerrard et al., 2005). In addition, inhibiting Notch and BMP-2 signaling may synergistically enhance neuronal differentiation more than suppressing either alone.

However, using these signaling molecules solely result in the mixed population of neural cells, indicating that there are most likely other signals found within the *in vivo* microenvironment that work in conjunction with each other to ensure a neuronal cell fate commitment.

3.2 Current protocols for DA differentiation

Numerous methods have been employed to differentiate mouse and human ESCs into neural cells. Mouse ESCs can be induced into neural progenitors by several methods, such as the use of retinoic acid (RA) treatment of embryoid body (EB) (Bain et al., 1995; Wichterle et al., 2002), a multistep-induction and selection culture (Lee et al., 2000), an adherent monoculture system in serum-free medium (Ying et al., 2003), and a co-culture with stromal cell types (Kawasaki et al., 2000, Barberi et al., 2003). Human ESCs can be induced into the neural lineages using similar methodologies: through the formation of EBs in suspension (Zhang et al., 2006), an adherent monoculture system (Benzing et al., 2006), with co-culture (Ueno et al., 2006) and through spontaneous differentiation of human ESCs (Zhang et al., 2001, Reubinoff et al., 2001). Each of these protocols has its pros and cons. In the following part, the most used procedures for neural differentiation of ESCs will be described.

3.2.1 Embryoid body formation

The well-studied system for neuronal differentiation of ESCs involves the formation of three-dimensional structures called embryoid bodies (EBs) that, to a limited extent, simulates embryonic development *in vivo* (Itskovitz-Eldor et al., 2000). EBs are spontaneously generated when ESCs are cultured in suspension cultures without LIF or serum, in either non-adhesive dishes or hanging drops. The cells in EB begin to differentiate into a heterogeneous

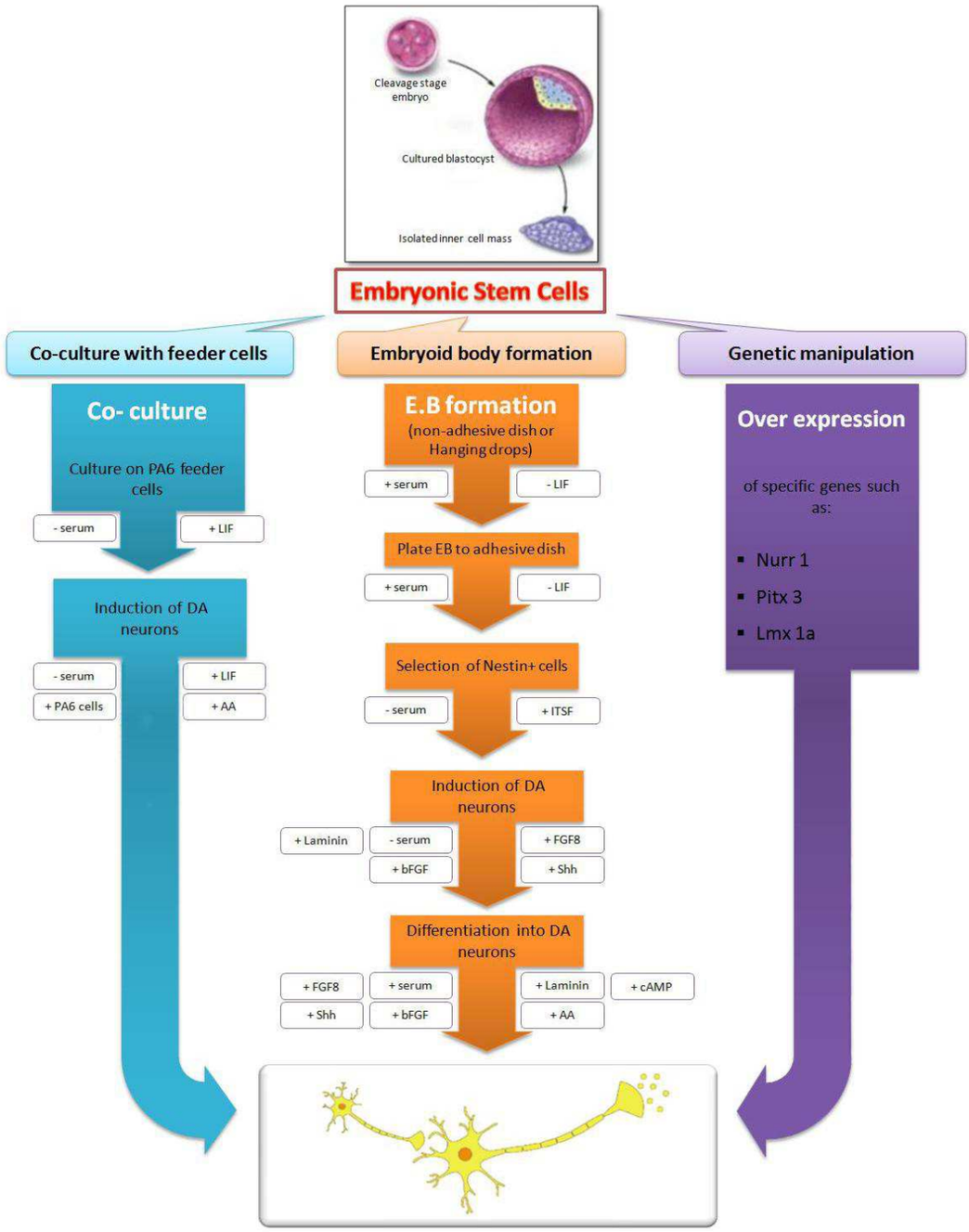

Fig. 2. Schemata depicting steps of three protocols by which ESCs are induced to adopt the DA Lineage.

population of progenitor cells that can form all cell types from the three germ layers, such as skeletal muscle, cardiac, hematopoietic and neuron-like cells. Therefore, spontaneous differentiation of EBs yields only a small fraction of cells with neural lineages. By using different morphogens and growth factors during EB formation, a higher fraction of neural cells can be produced (Figure 2).

After transfer of EBs from a low attachment plate into a normal adhesion plate, the EBs form neuroepithelial cells that organize into neural tube like rosettes. After dissociation of neuroepithelial cells and addition of neural differentiation medium, which consists of BDNF, GDNF, AMP, and ascorbic acid, DA differentiation begins 3–4 weeks after the initial treatment of ESCs (Yan et al., 2005).

EB formation method is still commonly used with the addition of the other supplementary to media such as growth factors. Bain *et al* were the first to show that RA can induce neural differentiation in EBs derived from mouse ESCs, i.e. a high proportion of the resulting cells expressed neuronal markers and had neuronal properties (Bain et al., 1995). In this work, EBs were cultured with RA for 4 days and then plated on laminin-coated dishes. The cells expressed neuronal markers beta-tubulin III and NF-M as well as neural-related genes such as transmitter synthesizing genes glutamic acid decarboxylase (GAD), TH, transmitter receptor subunits GluRs, and a cytoskeletal subunit, NF-L.

RA applied to ESCs can induce concentration-dependent differentiation of neural cells. Okada *et al* tested the effects of different concentrations of RA on the neural differentiation of mouse ESCs (Okada et al., 2004). Lower RA levels (10^{-8}M) were found to induce neural progenitor cells from ESCs, indicated by the high protein expression of the neural precursor marker nestin and low expression of neuronal and glial markers beta-tubulin III and GFAP, respectively. In contrast, high levels of RA (2×10^{-6} M) decreased the expression of nestin while increasing beta-tubulin III and GFAP levels. These results are consistent with other studies demonstrating differentiation of neural progenitors at high RA concentrations (Wichterle., 2002, Bain et al., 1996). RA also directs neural differentiation in human ESCs derived-EB cultures (Guan et al., 2001). The addition of RA and nerve growth-factor (NGF) increased the rate of neuronal cells that formed within human ESC-derived EBs (Schuldiner et al., 2001). However, RA is a strong teratogen and should therefore be used at lower doses to prevent toxicity.

One strategy to direct mouse and human ESCs into "midbrain dopaminergic" neurons is through formation of EBs, followed by the combined addition of Shh and FGF-8 and at a later stage ascorbic acid (AA) (Lee et al., 2000). Using this method, 34% of the resulting beta-tubulin III positive neurons derived from mouse ESCs were TH positive. To shorten induction protocol time, Lau *et al* cultured EBs in KO DMEM supplemented with EGF, FGF-2, and ascorbic acid (Lau et al., 2006). After 3 days, EBs were plated on gelatin-coated dishes and cultured with DMEM/F12 containing EGF, FGF-8, Shh, and ascorbic acid. Cells were subsequently cultured in Neurobasal medium with ascorbic acid. This method generated approximately 40% DA neurons, characterized by their expression of dopamine transporter DAT in 14 days. This chemically defined system for ESCs derivation of DA neurons may be advantageous compared to the co-culturing method, which will be discussed in the next part, as it reduces exposure to animal-derived components and allows for easier determination of factors that influence cell fate.

Another strategy to obtain "midbrain dopaminergic" neurons is the growth of EBs in a conditioned medium with a human hepatocarcinoma cell line followed by conventional serum- free culture in a medium containing bFGF (Schulz et al., 2004, Schulz et al., 2003), or by co-culturing them with telomerase-immortalized fetal midbrain astrocytes (Roy et al., 2006). EBs plated on tissue culture dishes and in the presence of serum-free ITSF medium showed induced differentiation toward dopaminergic precursors within 10 days. In the next step, the cells were transferred to polyornithine/laminin-coated dishes and exposed to a new medium supplemented with FGF2 and Shh (Roy et al., 2006). Withdrawal of these

factors, but the addition of BDNF, GDNF, and FBS, yields dopaminergic neurons that are TH positive. The majority of TH positive cells expressed simultaneously G-protein gated inwardly rectifying potassium channel type 2 (Sonntag et al., 2007), which is almost exclusively expressed in the membrane of DA neurons projecting to the dorsolateral putamen, and are functionally linked to dopamine D2 and GABAB receptors (Sonntag et al., 2007).

3.2.2 Co-culture with feeder cells

Another important strategy to enhance the differentiation toward neuron lineage is co-culture of ESCs with stromal cell lines such as PA6 (Kawasaki et al., 2000) and MS5 (Barberi et al., 2003). This effect of PA6 cells has been named the inductive factor stromal cell-derived inducing activity (SDIA) (Mizusekiet et al., 2003; Kawasaki et al., 2000). After screening various cell lines, Kawasaki *et al* found that PA6 stromal cells derived from mouse skull bone marrow is a potent inducer of neural differentiation from ESCs (Figure 2). In contrast to EB formation protocol, this method does not require growth in serum, the formation of EBs, or the selection of neural precursor cells. In this method, ESCs were co-cultured without serum on PA6 cells for 8 days in differentiation medium and for an additional 6-12 days in G-MEM supplemented with N2 and other components. Ninety-two percent of the colonies contained differentiated neurons positive for neural markers NCAM, nestin, and beta-tubulin III and MAP2 and only less than 2% of the colonies were positive for mesodermal and glial markers.

Co-culturing with stromal cell lines with some modifications has become a common strategy to differentiate ESCs into neural cells. In comparison to EB formation protocol, this method has fewer steps and is relatively easier and more reproducible for the generation of neural precursors and neuronal subtypes (Figure 2). Due to the risk of contamination with animal-derived components (Martin et al., 2005), some alternative approaches were recently developed to use instead of stromal feeders. Human amniotic membrane, the innermost layer of placenta has been used as an alternative (Ueno et al., 2006; Niknejad et al., 2008). This approach involves the co-culture of matrix layers of human amniotic membrane with human ESCs for neural induction. Fifteen days after culturing on amniotic membrane, human ESCs produced a population of cells that were greater than 85% nestin positive, and many of these formed rosette-like clusters. While this method eliminates the introduction of animal-derived products, identification of the factors involved in the regulation of neural differentiation, and overcoming inherent limitations in the scale up of processes involving cell co-cultures, need to be further addressed.

Kitajima *et al* devised a co-culture method for producing neurospheres using PA6 stromal cells (Kitajima et al., 2005). To induce neural spheres, ESCs were differentiated on PA6 stroma for 7 days, detached, dissociated, and cultured in growth medium with FGF-2 and EGF. The resulting neurospheres expressed multiple neural markers such as nestin, MAP2 and GFAP, indicative of a heterogeneous population of neural progenitors, mature neurons, and glial cells. Co-culture with PA6 cells for 0-13 days progressively increased the number of spheres generated in a time-dependent manner until day 11. The resulting neurospheres could be further propagated when switched to a serum-free culture and then differentiated into all three neural types. By changing the time of co-culture with PA6 cells, it was also possible to induce different proportions of neuronal and glial precursor cells. This system thus enabled the production of large numbers of spheres without utilizing EB formation.

Also, using MS5 instead of PA6 stromal cells efficiently induced neural differentiation of mouse ES cells, and the resulting cells were able to differentiate into more neuronal types (GABAergic, sertonergic, dopaminergic and cholinergic neurons) (Barberi et al., 2003).
Yue *et al* showed that primate ESCs can differentiate into dopaminergic neurons by coculture of ESCs with sertoli cells. Neurons that had been differentiated on sertoli cells were positive for Pax2, En1, and AADC; midbrain related markers and negative for dopamine-β-hydroxylase, a marker of noradrenergic neurons and could release dopamine *in vitro* when depolarized by KCL (Yue et al., 2006).

3.2.3 Genetic manipulation

At this time, EB formation protocol and co-culture with feeder cells typically lead to a mixed population of cell types. Moreover, to effectively use this approaches *in vitro* the dose, order of addition, and time of exposure to growth factors are all important parameters that must be optimized. This is a difficult task since many of the signals involved in regulating neuronal differentiation are only now being elucidated. It seems transfection of key genes such as important transcription factors (using conventional DNA delivery, lentiviral and adenoviral vectors, and homologous recombination) to ESCs is capable of inducing a specific neural lineage and can potentially increase the homogeneity of differentiated cells (Figure 2).
To enhance TH expression in neural stem cells, Sakurada *et al* used a retrovirus to overexpress Nurr1, a transcription factor belonging to the nuclear receptor super family that is expressed in midbrain DA neurons. This approach induced TH expression in nearly all infected cells *in vitro*. However, infected cells did not express detectable levels of DOPA, the dopamine precursor whose production is catalyzed by TH, and functional production of DOPA was only detected when the cells were differentiated with retinoic acid. Even though the resulting population uniformly expressed TH, the frequency of neuronal markers within these TH positive cells was still low (Sakurada et al., 1999). These results implicate a need for additional manipulations to increase neuronal maturation and the functional production of dopamine.
Similar results were observed in neural stem cells derived from E13/E14 rat foetal brain tissue, in which Nurr1 overexpression led to an increase in TH expression (Kim et al., 2003). However, infected cells did not mature. Park *et al* used retroviral vectors that co-expressed Nurr1 and a second transcription factor to force neuronal differentiation. They examined the bHLH transcription factors Mash1, Ngn1, Ngn2 and NeuroD1, all known to induce neuronal differentiation (Kageyama& Nakanishi, 1997), and they reported that the induction of TH expression depended on which bHLH transcription was co-expressed with Nurr1. Ngn1, Ngn2 and NeuroD1 decreased the Nurr1-induced expression of TH, whereas Mash1 overexpression not only maintained expression but also increased the fraction of cells that expressed the neuronal marker Map2ab. Importantly, they showed that the combined expression of Mash1 and Nurr1 yielded neurons with electrophysiological properties similar to those of mature DA neurons (Park et al., 2006a).
Furthermore, Park *et al* showed that when grafted into Parkinsonian rats, cells co-expressing Nurr1 and Mash1 reversed behavioral deficits. Intriguingly, a retrovirus encoding Shh, the anti-apoptotic protein Bcl-xl, and Nurr1 produced functionally mature dopaminergic neurons similar to the Mash1 vector studies (Park et al., 2006b). These studies demonstrate the potency of genetic manipulation and highlight the fact that significant additional work

will be needed to explore whether extracellular signal combinations can achieve similar results.

In addition to its role in directing differentiation towards a DA phenotype, Nurr1 synergizes with Pitx3 to promote terminal maturation of midbrain DA neurons from both mouse and human ESCs (Martinat et al., 2006). Lentiviral vectors carrying Nurr1, Pitx3, Lmx1b, or En1 were introduced at the neural precursor stage after induction into EBs from mouse ESCs. The combined transduction of Nurr1 and Pitx3 dramatically induced the expression of the late marker DAT (dopamine transporter), but not TH. Only Nurr1 alone induced expression of TH.

The two predominant methods developed on mouse cells, transfection of mouse ESCs with specific factors such as Nurr-1 and Lmx1a as well as co-cultures with stromal cells, have been translated to human ESCs to direct DA neuron differentiation. Similar results were found after human ES cells were transduced with lentiviral vectors carrying both Nurr1 and Pitx3 at the neural precursor stage as well as co-cultured with stromal cells (Martinat et al., 2006). Nurr1 and Pitx3 together promoted the maturation of midbrain DA neurons and led to an increase in TH positive cells. In electrophysiological analysis, differentiated human ESCs displayed basic neuronal characteristics such as action potentials, burst firing, and miniature spontaneous excitatory postsynaptic currents. Transplantation of these cells into a mouse model of PD improved some motor ability 6 weeks post-transplantation; however, there was limited maturation of the engrafted human ES-derived cells, as assessed by low expression of TH

4. ESCs in PD: hype or hope

Despite all of the advantages of ESCs for cell replacement therapy, some pitfalls must be overcome before ESCs are used therapeutically for PD. The major limitation is ethical issues concerning to their embryonic origin. Human embryos are most often destroyed as the stem cells are harvested. Hence the question: Can we intentionally kill a developing human being at this stage to expand scientific knowledge and potentially provide medical benefit to others? As a result, some regulations limit funding of research to the initial set of derived human ESCs, strictly withholding support for the study and derivation of new stem cells lines. However, creating new stem lines by deriving human ESCs from single blastomeres (Klimanskaya et al., 2006), without destroying the embryo was a promising result to bypass the ethical problem of ESCs.

The other challenge in employing ESCs for developmental biology research and their possible application in cell replacement therapy is to direct their wide differentiation potential into specific neural cell lineages. The most important concern of the mentioned protocols of ESCs differentiation toward specific neural lineages is the nonspecific generation of cell populations derived from the three germ layers in the total cell population. In all protocols, the presence of mesodermal- and endodermal-originated cell lineages is unavoidable, which is undesirable for further application in regenerative medicine. Hence, understanding the coordination and roles of intrinsic factors with extrinsic factors will be a critical step to direct ESCs differentiation into dopaminergic neuronal cells.

In addition to mesodermal and endodermal lineages, current protocols result in heterogonous population of glia and neural cells, such as serotonergic, GABAergic and noradrenergic. To date, it is uncertain which of these ingredients are of positive or negative impact on the clinical effectiveness of the transplants. For example in transplantation of

foetal mesencephalon which is a mixture of all neural cell types homing to the foetal midbrain, containing serotonergic neurons might be responsible for dyskinetic side effects. On the other hand, it has been shown that foetal midbrain-derived astrocytes have beneficial effects on in vitro differentiation of ESCs into DA neurons. Transplanting a mixture of dopaminergic precursors/neurons and midbrain-specific astrocytes might thus be more effective than a purified DA cell source. No information is available on the effect of GABAergic or noradrenergic neurons also present in midbrain transplants. Therefore, further studies will be required to investigate the effects of the other constituents of neural cells other than DA neurons on dopaminergic differentiation of ESCs and after grafting in the animal model of PD.

As mentioned, current differentiation protocols rely on the use of animal products (e.g. PA6 is a mouse stromal cell line) and thus carry the potential to induce disease transmission through contamination with bacteria, viruses or other infectious agents, such as those responsible for transmissible spongiform encephalopathy. Therefore, animal products should, where possible, be replaced by components of human origin and defined and feeder-free conditions should be developed for differentiation of ESCs into dopaminergic neurons if the cells are going to be implanted in patients.

The other unresolved problem is whether the grafts will be affected by the PD process. The results of foetal mesencephalic tissue transplantations provide evidence that PD pathology might propagate from host to graft (Kordower et al., 2008a; Li et al., 2008; Brundin et al., 2008, Kordower et al., 2008b). However, it seems that at least one decade is required for the development of Lewy bodies (LBs) in the grafted cells (Kordower et al., 2008a; Mendez et al., 2008). This issue should be noticed in the animal model of PD.

Rejection of grafts is an important issue in cell replacement therapy, particularly for ESCs which are transplanted as an allograft. Although immune responses to brain allografts are moderate and survival can be obtained even without immunosuppression (Freed et al., 2001), most investigators prefer to use immunosuppressive agents for 6 to 12 months after transplantation (Brundin etal., 2000, Mendez et al., 2005; Olanow et al., 2003). Another way to keep away from immune reactions after transplantation is the using of therapeutic cloning. It has been shown that genetically identical ESCs derived DA neurons, generated by transfer of autologous nuclei from fibroblasts, improve functional deficits without immune reaction in PD mice (Tabar et al. 2008). However, it should be evaluated whether genetically modified cells would be acceptable in a clinical protocol.

Tumor formation remains another obstacle concerning the transplantation of ESCs that has to be eliminated before human ESCs can be safely applied in PD. Because life expectancy is almost normal in PD patients, even a minor risk of tumor formation associated with cell replacement therapy is unacceptable in this disorder. To improve safety, it might be necessary to engineer ESCs with regulated suicide genes or to use cell sorting to eliminate cells that could give rise to tumors.

In the end, on the basis of the available experimental data, ESCs are promising source for the cell replacement therapy. Although much more studies will be required to overcome the mentioned challenging issues, it seems it is now possible to start defining a road map including the main steps towards clinical application of ESCs in PD.

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Parkinson's disease (PD) results primarily from the death of dopaminergic neurons in the substantia nigra. Current PD medications treat symptoms; none halt or retard dopaminergic neuron degeneration. The main obstacle to developing neuroprotective therapies is a limited understanding of the key molecular mechanisms that provoke neurodegeneration. The discovery of PD genes has led to the hypothesis that misfolding of proteins and dysfunction of the ubiquitin-proteasome pathway are pivotal to PD pathogenesis. Previously implicated culprits in PD neurodegeneration, mitochondrial dysfunction, and oxidative stress may also act in part by causing the accumulation of misfolded proteins, in addition to producing other deleterious events in dopaminergic neurons. Neurotoxin-based models have been important in elucidating the molecular cascade of cell death in dopaminergic neurons. PD models based on the manipulation of PD genes should prove valuable in elucidating important aspects of the disease, such as selective vulnerability of substantia nigra dopaminergic neurons to the degenerative process.

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