

Efficient Induction of Dopaminergic Neurons from Embryonic Stem Cells for Application to Parkinson's Disease

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For cell replacement therapy of neurodegenerative diseases such as Parkinson's disease (PD), methods for efficiently generating midbrain dopaminergic (DA) neurons from embryonic stem (ES) cells have been investigated. Two aspects of DA neuron generation are considered: genetic modification and manipulation of culture conditions. A transcription factor known as critical for development of DA neurons, Nurr1, was introduced into ES cells to see how they facilitate the generation of DA neurons from ES cells. Also, two culture procedures, the 5-stage method and stromal cell-derived inducing activity (SDIA) method, were used for ES cell differentiation. Using the 5-stage method, we and others previously demonstrated that Nurr1-overexpressing ES cells, under treatment of signaling molecules such as SHH and FGF8 followed by treatment of ascorbic acid, can differentiate into DA neurons with a high efficiency ($> 60\%$ of TH⁺/Tuj1⁺ neurons). Furthermore, using the SDIA method with treatment of signaling molecules, we found that Nurr1-overexpressing ES cells can differentiate to DA neurons with the highest efficiency ever reported ($\sim 90\%$ of TH⁺/Tuj1⁺ neurons). Importantly, our semi-quantitative and real-time PCR analyses demonstrate that all known DA marker genes (e.g., TH, AADC and DAT) were up-regulated in Nurr1-overexpressing ES cells when compared to the naïve ES cells. These cells produced increased dopamine compared to naïve D3 cells after differentiation. In the *in vivo* context after transplantation, the genetically modified ES cells also showed the highly increased dopaminergic neuronal phenotypes. Thus, the combination of genetic engineering and appropriate culture conditions provides a useful tool to generate a good cell source from ES

cells for cell replacement therapy of degenerative diseases such as PD.

Key Words: Embryonic stem cells, differentiation, dopaminergic neurons, parkinson's disease, neurodegenerative disorders

Parkinson's disease (PD) is a very common neurodegenerative disorder characterized by the selective degeneration of dopaminergic (DA) neurons in the substantia nigra (A9) of the mid-brain.^{1,2} A9 DA neurons innervate the dorsal striatum and this nigrostriatal pathway controls voluntary movements. Dysfunction of A9 DA neurons leads to tremor, rigidity and hypokinesia. In contrast to A9 DA neurons, DA neurons of the ventral tegmental area (A10) are relatively unaffected in PD and innervate the ventral striatum and neocortex. This mesolimbic pathway controls emotion and reward. Dysfunction of this pathway has been implicated in psychiatric disorders such as schizophrenia and drug addiction.³

Pharmacological treatment of PD using L-DOPA has limitations because it later leads to a side effect.⁴ To overcome this limitation, transplantation therapy with various donor cells has been investigated. Although grafts of fetal ventral mesencephalon looks promising for the treatment of PD,⁵⁻⁷ its clinical application also has limitations due to technical and ethical problems in obtaining large numbers of human fetal brain tissue.

Embryonic stem (ES) cells are derived from the inner cell mass of mammalian blastocysts,^{8,9} can proliferate extensively *in vitro* while maintaining an undifferentiated state and can differentiate into most cell types under certain conditions.^{10,11} Thus,

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these properties of ES cells make them a good source for cell replacement therapy as well as a useful tool to analyse critical steps of cell development. Several laboratories have been successful in developing methods to induce these ES cells to differentiate into DA neuronal phenotypes for application to PD.

Methods for Efficiently Generating Midbrain DA Neurons from ES Cells

Manipulation of culture conditions

5 stage method

Several laboratories have demonstrated that phenotypes characteristic of midbrain DA neurons can be efficiently induced from mouse and primate ES cells. Two aspects of DA neuron generation have been considered: manipulation of culture procedures^{12,13} and genetic modification.^{14,15} Two culture procedures for efficient *in vitro* differentiation of mouse or primate ES cells into the neuronal phenotype have recently been established (Fig. 1). One is a 5 stage method developed by Lee *et al.*¹³ It consists of EB formation (4 days), selection of neural precursors (7-10 days), expansion of neural precursors (4 days) and differentiation into neurons (11-15 days). This method can generate as much as 34% of tyrosine hydroxylase (TH)-positive cells among Tuj1-positive neurons from mouse ES cells in the presence of signaling

molecules such as sonic hedgehog (Shh), fibroblast growth factor (FGF)-8, and ascorbic acid.

SDIA method

Another efficient *in vitro* method for DA neuron induction is the co-culture method with PA6 feeder cells which have the stromal cell-derived inducing activity (SDIA).¹² PA6 cells are derived from mouse skull bone marrow. This procedure also efficiently induces a high proportion of TH⁺ neurons (~30% of Tuj1⁺ neurons) from mouse ES cells. This method has several additional advantages over other EB-based methods including the five-stage method: (1) it does not depend on EB formation and selection of neural precursors, (2) the whole procedure takes approximately half the amount of time as the five-stage method, and (3) it shows reduced variability and higher yield of TH⁺ neurons in multiple ES cells.¹⁶ Importantly, this PA6 co-culture method was successfully applied for efficient generation of TH⁺ neurons from primate ES cells,¹⁷ suggesting that ES cells from different species including human may be coaxed to differentiate into DA neurons using this procedure.

Genetic Modification of ES Cells

Promoter selection

In addition to manipulation of culture procedures, it is also possible to successfully facilitate

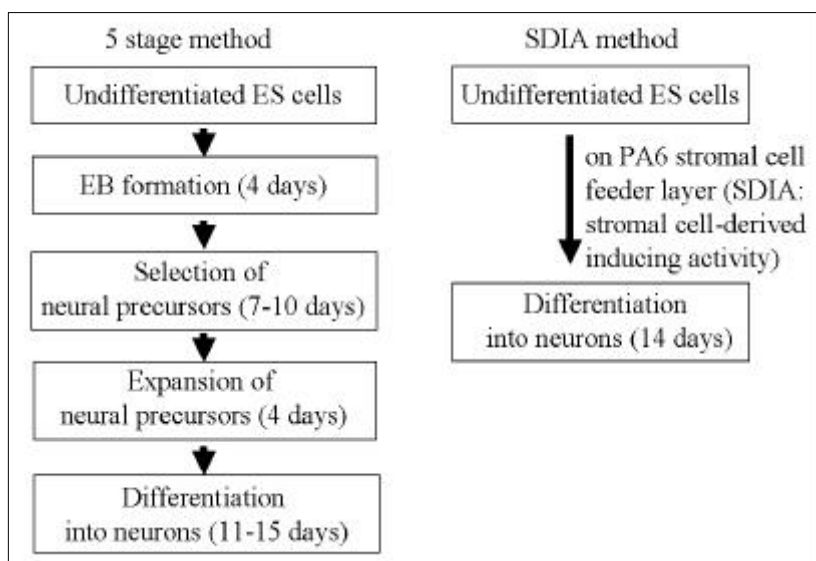


Fig. 1. Two *in vitro* differentiation methods for generation of neurons from mouse or primate ES cells.

the differentiation of ES cells to certain lineages by genetic manipulation consisting of the specific activation of key fate-determining transcription factors through viral infection or stable plasmid transfection.^{18,19} For genetic modification of ES cells by exogenous transgene expression, different viral and cellular promoters should be tested because in ES cells the relative strengths of various promoters are different. Thus, in each mouse or primate ES cells, it is first necessary to determine the optimal promoter system(s) for their genetic manipulation. For example, Chung *et al.*²⁰ reported that the human polypeptide chain elongation factor 1alpha (EF) promoter and the CMV immediate early enhancer fused to the chicken β -actin promoter (CBA) show robust activities in two different mouse ES cell lines, whereas the CMV promoter, in contrast to its strong activity in most cell lines, has only a marginal activity in mouse ES cells.

Introduction of genes

Recent data from our and other groups demonstrate that forced expression of Nurr1, a transcription factor critical for the development of midbrain DA neurons²¹⁻²³ can greatly facilitate the induction of DA neurons from mouse ES cells using the 5-stage protocol.^{14,15} It is likely that genetic modification of ES cells can be combined with certain culture conditions to efficiently induce certain cell lineage(s). Indeed, Nurr1-overexpressing mouse ES cells (N2), when combined with treatment of signaling molecules (Shh, FGF8, and ascorbic acid), could differentiate into DA neurons with a high efficiency (>60% of TH⁺/Tuj1⁺ neurons) using the 5-stage protocol (Fig. 2).^{14,15} This increase in TH⁺ cells was accompanied by an increase in other DA markers such as AADC and DAT, as well as increased DA production.^{14,15} Finally, these DA neurons showed functional recovery when transplanted in an animal model of PD.¹⁴

Also, a simultaneous use of Nurr1, signaling molecules and SDIA in neuronal differentiation of mouse ES cells highly increased the proportion of DA neurons. Very recently, we found using the SDIA method plus signaling molecules that N2 cells can differentiate to DA neurons with the

highest efficiency ever reported (~90% of TH⁺/Tuj1⁺ neurons) (Fig. 2).²⁴ Importantly, these N2-derived TH⁺ neurons were found to coexpress the midbrain DA markers such as AADC and DAT, but not GABA or 5-HT. Furthermore, these neurons revealed up-regulation of DAT, AADC, Girk, calbindin, AHD2 and Pitx3 mRNAs by semiquantitative and real-time PCR analyses compared to naïve D3-derived neurons. Taken together, these results suggest that the TH⁺ neurons are midbrain DA neurons. These N2-derived neurons also showed high level of DA production inside the cells and efficiently released DA into the conditioned medium (>14 fold) or KCl-challenged medium (>4 fold) when compared to wild type D3, indicating these TH⁺ neurons are functional DA neurons (Fig. 3).

Increase of neuron numbers from mouse ES cells cultured on PA6 was evident by transgene expression of Nurr1.²⁴ When we counted Tuj1⁺ neurons, there was a significant increase in number of β -tubulin⁺ cells in N2 cell-derived neurons when compared with D3 cell-derived neurons. mRNA level of β -tubulin was also increased about 3 fold in N2 when compared to D3. Signaling molecules did not significantly affect neurogenesis. It has been reported that Nurr1 can induce cell cycle arrest and a highly differentiated cell morphology in dopamine MN9D cells.²⁵ Nurr1 is also essential for the survival of DA precursor neurons as well as the induction of the DA phenotype.²² In spite of this fact, the effect of

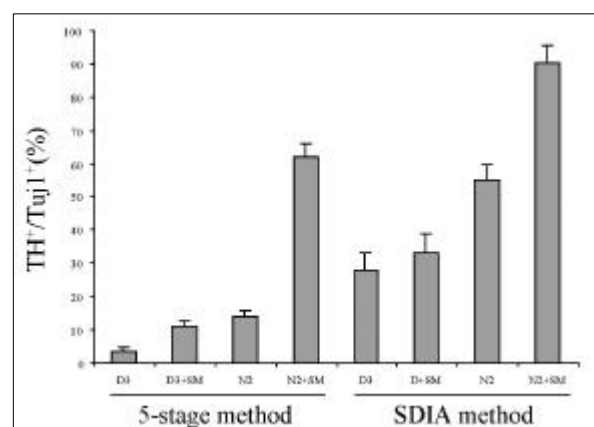


Fig. 2. Efficient induction of DA neurons by Nurr1 in the presence of signaling molecules following 5-stage or SDIA method. D3, naïve ES cells; N2, Nurr1-overexpressing ES cells; SM, signaling molecules.

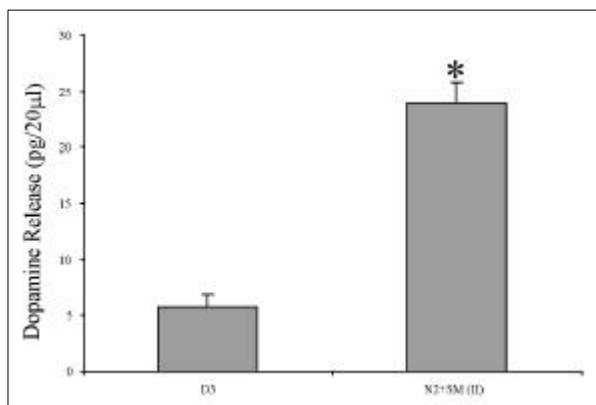


Fig. 3. DA level in 50 mM KCl-challenged media of D3- and N2-derived neurons were assayed by HPLC. D3, naïve ES cells; N2+SM (II), Nurr1-overexpressing ES cells treated with signaling molecules during days 5-9 followed by ascorbic acid treatment during days 9-14. Each group represents an average of 6 samples. Fisher's PLSD posthoc analysis was performed with a significance level of 0.05. * $p < 0.05$ compared to D3.

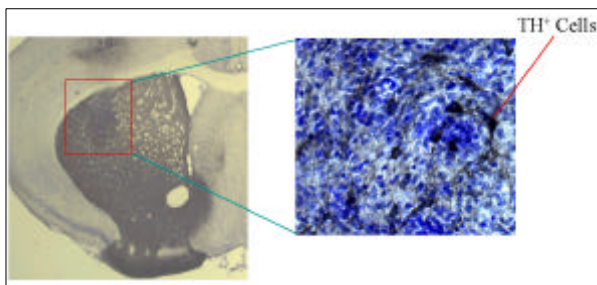


Fig. 4. Efficient *in vivo* integration/generation of TH⁺ neurons from N2 cells after transplantation in the mouse striatum. N2-derived cells were transplanted into the mouse striatum at *in vitro* differentiation day 9, and the animals were sacrificed 4 weeks after transplantation for histological analysis. Grafts of N2 cells in naïve mouse striatum were analyzed by TH/Nissl staining.

Nurr1 alone on neurogenesis from mouse ES cells using 5-stage protocol was not significant although they showed a slight increase in β -tubulin⁺ cell numbers.¹⁵ However, in the context of SDIA, Nurr1 promoted a significant increase in neuronal population. Any factor in PA6 cells in conjunction with Nurr1 may exert its role in neurogenesis although it is unclear.

Transplantation

In an effort to translate the *in vitro* results into

in vivo context, we transplanted partially differentiated cells from N2 under optimal condition into the mouse striatum. The mice were analyzed four weeks after transplantation. We found that many TH⁺ neurons were integrated/generated from the graft of N2 cells by TH/Nissl staining (Fig. 4).²⁴ In contrast, about one tenth of TH⁺ neurons were observed in the control graft of D3 cells. In co-staining with antibodies against TH and NeuN, a similar result was also observed. Thus, these results suggest that our *in vitro* optimal condition for efficiently generating DA neurons can be translated into the *in vivo* cell transplantation study for the potential treatment of neurodegenerative diseases such as PD.

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