Transplantation and functional analysis of mouse embryonic stem cell-derived dopamine neurons in a Parkinsonian rat model

Yoon Hee Cho

Department of Medical Science
The Graduate School, Yonsei University

Transplantation and functional analysis of mouse embryonic stem cell-derived dopamine neurons in a Parkinsonian rat model

Directed by Professor Jin Woo Chang

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Yoon Hee Cho

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This certifies that the Doctoral Dissertation of Yoon Hee Cho is approved

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Thesis Supervisor : Jin Woo Chang

Thesis Committee Member#1 : Dong Wook Kim

Thesis Committee Member#2 : Bae Hwan Lee

Thesis Committee Member#3 : Young Ho Sohn

Thesis Committee Member#4 : Jong Hee Chang

The Graduate School

Yonsei University

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많은 부족함을 느꼈음에도 이 논문이 완성될 수 있었던 것은 정말 많은 분들의 도움이 있었기 때문입니다. 오랜 시간 동안 신경외과 실험실에 있었지만, 마무리를 지을 즈음 되돌아보니 아쉬움과 후회가 앞섭니다. 부족한 저를 옆에서 지켜 봐 주시고 도움을 아끼지 않으셨던 장진우 지도교수님께 우선 감사를 드립니다. 바쁘신 중에도 많은 관심을 가져주시고 지원을 아끼지 않으셨던 교수님의 따뜻한 은혜에 진심으로 감사 드립니다. 또한 무엇보다도 부족한 저에게 옆에서 항상 힘이 되어 주시고, 격려와 자문을 아끼지 않으셨던 김동욱 교수님께도 깊은 감사를 드립니다. 제가 실험실 생활을 처음 시작할 즈음 많은 도움과 격려, 조언을 주셨던 이배환 교수님께도 이 지면을 빌어 깊은 감사를 드립니다. 이 논문이 완성되기까지 바쁘신 와중에도 자문을 해주신 손영호 교수님과 장종희 교수님께 감사 드립니다. 실험실 생활을

처음 할 무렵 옆에서 항상 힘이 되어주고 자신의 일처럼 도움을 주었던, 지금은 다른 곳에서 또 다른 인생을 열심히 가꾸어 가고 있는 동생 미파에게 고마움을 전합니다. 부족한 저에게 옆에서 조용히 도움을 아끼지 않았던 믿음직한 용섭이에게 따뜻한 고마움을 전합니다. 여러 해 동안 실험실 생활을 하며 고민을 함께 해준 이경희 선생님, 부족한 저를 친 언니, 누나처럼 따라주던 든든한 동생들 은희와 명희, 재형이에게 고마움을 전합니다. 이 논문에 많은 도움을 주었던 대성이와 권오규 선생님, 그리고 조명수 박사님에게 고마움을 전합니다. 이렇게 많은 도움을 받았음에도 그만큼 많은 도움이 되어주지 못한 것에 또한 미안한 마음을 전합니다.

제 인생의 처음부터 지금까지, 그리고, 앞으로도 변함없이, 말로 다 할 수 없는 무한한 사랑으로 든든한 버팀목이 되어주시는 사랑하는 아빠, 엄마께 진심으로 머리 숙여 감사 드립니다. 저의 부족함을 아시기에 옆에서 늘 힘이 되어 주시길 아끼지

앓으셨던 부모님께 이 논문을 바칩니다. 사랑합니다. 옆에서 격려와 사랑으로 항상 나의 편이 되어주는 사랑하는 남편 정현에게 진심으로 감사한 마음을 전합니다. 부족한 며느리에게 격려와 따뜻한 말씀을 아끼지 않으시는 아버님, 어머님께 감사 드립니다. 제 인생에 가장 소중한 보물 민지와 나윤이, 사랑하는 가족 은영 언니, 형부, 윤정, 정빈, 윤수, 영욱, 동엽, 지윤이 있어서 이런 작은 결실을 맺게 되었음을 또한 감사 드립니다. 앞으로 어떤 삶이 저에게 펼쳐질지 모르지만 부족함을 깨닫고 겸손해 질 수 있었던 지금의 시간들이 있었기에 한 걸음 더 나아가려 합니다. 이런 시간을 함께 해준 모든 이들에게 감사의 마음을 전합니다.

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조 윤희

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Abstract

Transplantation and functional analysis of mouse embryonic stem cell-derived dopamine neurons in a Parkinsonian rat model

Yoon Hee Cho

Department of Medical Science
The Graduate School, Yonsei University

(Directed by Professor Jin Woo Chang)

Embryonic stem cells (ESCs) are good for application such a Parkinson's disease because it primarily involves the degenerative loss of a specific cell type, namely the midbrain dopaminergic (DA) neurons in the substantia nigra. To test the *in vivo* effect of a high yield of DA neurons (90% of total

neurons) which had been generated from a genetically modified mouse ESC line, N2, the cells were transplanted into a rat model of Parkinson's disease (PD). The PD animals grafted with N2-derived cells showed significant behavior improvements compared with sham controls from 2 weeks posttransplantation, whereas animals with naïve D3-derived cells (~28% DA neurons of total neurons) showed only a modest recovery. Furthermore, hyperactivity observed in the subthalamic nucleus (STN), pedunculopontine nucleus (PPN), and substantia nigra pars reticulata (SNpr) of PD rat models was dramatically reduced by the grafting of N2-derived cells. The number of DA neurons in the striatum which originated from N2 grafting was much higher compared to that from D3 grafting, and the neurons efficiently released DA in the brain, showing a good correlation with behavioral recovery.

Key words: Embryonic stem cells, Differentiation, Dopamine neurons, Parkinson disease, Behavior recovery

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Ⅰ. INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the progressive loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) and a reduction in striatal dopamine.[1,2] This leads to a variety of symptoms including the primary motor deficits of tremor, bradykinesia, and rigidity, as well as other non-motor problems. In PD, the progressive loss of DA neurons in the SNpc leads to impaired information processing in the basal ganglia. More specifically, it is thought that the reduction in the dopamine level is responsible for the imbalance in the activities of the direct and indirect pathways from the striatum to the basal ganglia output structures, the pars reticularta of the substantia nigra (SNpr),

and the internal portion of the globus pallidus (GPi).[3,4] The increased activity of basal ganglia output structures in the dopamine-depleted state may be partially due to an elevation of the excitatory drive from the subthalamic nucleus (STN). The reduction of subthalamic neuronal output has been found to reverse the behavioral effects in Parkinsonian rats, primates, and humans.[5,6,7,8,9,10]

Deep brain stimulation of the GPi and the subthalamic nucleus (STN), which is believed to relieve the motor symptoms by leading to depolarization blockage of neurons in the area.[10,11] Although neurosurgical treatment, deep brain stimulation, has been found to be beneficial for some patients with advanced PD, the prevailing strategy for the treatment of PD is pharmacological. Current approaches for the treatment of PD include the treatment with combined L-DOPA and carbidopa, which increase the synthesis and release of dopamine. These treatments are particularly effective in alleviating the akinesia and the rigidity during early stages of the disease. However, as the disease progresses, less dopamine neurons are available to synthesize dopamine, the effectiveness of the treatment decreases and L-DOPA-induced dyskinesia appears.[11]

Thus, an alternative approach for restoration of the damaged DA system is transplantation of DA-synthesizing cells. Cell transplantation to replace lost neurons, such as the case of PD is based on as follows: the predominent symptoms of PD are dependent on the loss of the DA neurons in the nigrostriatal pathway: and DA neurons grafted into the dopamine-deficient striatum can replace those neurons lost as a result of the disease process and can reverse, at least in part, the major symptoms of the disease. Therefore, cell transplantation is a new approach for the treatment of progressive neurodegenerative disease. Many studies have reported that fetal brain cells can relieve the symptoms of PD.[12,13] However, this approach also has

limitations, including an ethical issue and the technical difficulty of obtaining large amounts of fetal brain tissue.[14,15,16,17,18] To obtain DA-synthesizing cells from other sources, many scientists have tried to develop protocols for inducing DA neurons from embryonic stem cells (ESCs).[19,20,21,22,23,24,25,26]

ESCs are pluripotent and capable of self-renewal. ESCs are derived from the inner cell mass of mammalian blastocysts. Because of their ability to proliferate indefinitely *in vitro* while maintaining an undifferentiated state with their developmental potential to differentiate into most cell types, ESCs are not only useful to analyze critical steps of cell development but also as a potential source for cell replacement therapy.[22,28,29,30,31,32] These ESCs are good for application such a PD because it primarily involves the degenerative loss of a specific cell type, namely the midbrain DA neurons in the substantia nigra.[33,34] For the purpose of applying the ESCs to PD, many researchers have tried to develop protocols by which ESCs from many species can differentiate into DA neuronal phenotypes.[19,20,21,22,23,24,25,26,35] This study analyze of ESCs-derived DA neuron application for hemi-parkinsonian rat model. Several laboratories have demonstrated that phenotypes characteristic of midbrain DA neurons can be efficiently induced *in vitro* and/or *in vivo* from mouse and primate ESCs. The five-stage method is one of most successful *in vitro* induction procedures in which mouse ESCs are first grown to form embryoid bodies (EB), followed by selection and expansion of $nestin^+$ neural precursors, and differentiation into neural subtypes.[20] Another efficient *in vitro* method for DA neuron induction is the co-culture method with PA6 feeder cells that have stromal cell-derived inducing activity (SDIA).[21] This method can generate as much as 34% of tyrosine hydroxylase $(TH)^+$ cells among $Tuj1^+$ neurons, in the presence of signaling molecules such as sonic hedgehog (Shh), fibroblast growth factor (FGF) 8, and ascorbic acid. Recently reported that, in the presence of signaling

molecules, the co-culturing of Nurr1-expressing mouse ESCs (N2 or N5) with PA6 stromal cells can synergistically generate enough DA neurons to compose up to 90% of total neurons.[36] Recent data from this and other groups demonstrated that forced expression of Nurr1, a transcription factor critical for the development of midbrain DA neurons,[22,23] can greatly facilitate the induction of DA neurons from mouse ESCs.[25,26] These cells show that the high yield of DA neurons efficiently functions in a Parkinsonian rat model.

The present study demonstrates that DA-synthesizing cells from mouse ESCs that a very high proportion of midbrain DA neurons (up-to 90% of $Tuj1^+$ neurons) can be generated from Nurr1-overexpressing mouse ESCs using the optimized PA6 co-culture method within a short period of time (~14 days) can functionally integrate into host rat brain tissue and can also lead to behavioral recovery in a rodent model of PD. To investigate the transplantation effects of ESCs in a rat PD models, we evaluated the behavioral test such as a forepaw adjusting step test and an apomorphine induced rotation test at pre-transplantation and post-grafting mouse ESCs in the parkinsonian rat models. We analyzed histological assessment for transplanted TH^+ cells by immunohistochemistry.

We studied analysis of dopamine, implanting with microdialysis probe in the ipsilateral striatum of experimental animals.

To electrophysiologically investigate the transplantation effect, we examined the firing rates and firing patterns in the PPN, SNpr, and STN by extracellular single-unit microelectrode recording. In PD, the progressive deficit of DA cells in the SNpc results in hyperactivity in the SNpr, STN, and PPN due to impaired information processing in the basal ganglia.

Ⅱ. MATERIALS AND METHODS

1. In vitro Differentiation of ESCs

Undifferentiated mouse ESCs (the wild-type D3 and Nurr1-expressing N2) were maintained on gelatin-coated dishes in DMEM (Gibco, Rockville, MD, USA) supplemented with 2mM glutamine (Gibco), 0.001% 2-mercaptoethanol (Gibco), 1x non-essential amino acids (Gibco), 10% donor horse serum (Sigma), and 2000U/ml human recombinant leukemia inhibitory factor (LIF; R & D Systems, Minneapolis, MN, USA). To differentiate ESCs, PA6 cells were plated on gelatin coated culture dishes to make a uniform feeder monolayer 1 day before the addition of D3 or N2 cells. ESCs were then allowed to differentiate on the PA6 feeder cells for 14 days. ES differentiation medium I [G-MEM medium (Glasgow minimum essential medium, Gibco) supplemented with 10% knockout serum replacement (KSR, Gibco), 0.1 mM nonessential amino acids (Gibco), 1 mM sodium pyruvate (Sigma), 0.1 mM 2- mercaptoethanol (Gibco) and PEST (Gibco)] was used for 8 days and then replaced with ES differentiation medium II [G-MEM medium (Gibco) supplemented with N-2 supplement (Gibco), 0.1 mM nonessential amino acids (Gibco), 1 mM sodium pyruvate (Sigma), 0.1 mM 2- mercaptoethanol (Gibco) and PEST (Gibco)] for an additional 6 days. The culture medium was changed on day 4 and every other day thereafter. During differentiation of N2 cells, signaling molecules such as Shh, FGF 8 were treated during days 5-9 and ascorbic acid was added during days 9-14.[36]

2. Immunostaining

After 14 days of differentiation on PA6 feeder layer, ESCs were fixed with 4% formaldehyde for 30 minutes, rinsed with phosphate-buffered saline (PBS), and then incubated with blocking buffer (PBS, 10% normal donkey serum {NDS} or normal goat serum {NGS}, and 0.1% Triton X-100) for 10 minutes. Cells were incubated overnight at 4℃ with primary antibodies dilutes in PBS containing 2% NDS or NGS. The following antibodies were used: rabbit anti-β-tubulin (1:2,000; Covance, Richmond, CA, USA), sheep anti-TH (1:200; Pel-Freez, Rogers, AK, USA).

The coverslips were washed with PBS and then incubated with fluorescent labeled secondary antibodies {Alexa Fluor 488 (green) or Alexa Fluor 568 (red)-labeled donkey/goat IgG (1:500 Molecular Probes, Invitrogen, Carlsbad, CA) in PBS with 2% NDS or NGS for 30 minutes at room temperature. The coverslips were rinsed for 3 x 10 minutes in PBS and mounted onto slides using Gel/Mount (Biomeda Corp., Foster City, CA). Cells were examined using a Leica TCS/NT conforcal microscope equipped with krypton, krypton/argon, and helium lasers. Cells were counted according to the protocol to set up previously [26] with a slight modification.

3. Semiquantitative reverse transcription polymerase chain reaction analysis

The procedure was performed as described previously.[36] After differentiation, total RNA from ESC-derived neurons was extracted using the Easy-Spin kit (Intron, Korea) and DNase I treatment to avoid genomic contamination. Total RNA (1 μg each) was reverse transcribed with power cDNA synthesis kit (Intron, Korea) as manufacture's instruction. Polymerase Chain Reaction

(PCR) conditions were optimized, and linear amplification range was determined for each primer by varying annealing temperature and cycle number. PCR products were identified by size.

Primer sequences were: Oct4(5'-CGTTCTCTTTGGAAAGGTGTTC-3', 5'-ACACTCGGACCACGTCTTTC-3'), Sox1(5'-CAATGCGGGGAGGAGAAGTC-3', 5-'CTCTGGACCAAACTGTGGCG-3'), TH(5'-TTGGCTGACCGCACATTTG-3';5'-ACGAGAGGCATAGTTCCTGAGC-3'), DAT(5'-CAGAGAGGTGGAGCTCATC-3',5'-GGCAGATCTTCCAGACACC-3'), GAPDH(5'-GGCATTGCTCTCAATGACAA-3',5'-AGGGCCTCTCTCTTGCTCTC-3'). For semiquatitative RT-PCR, PCR reactions were carried out with 1x IN Reaction Buffer (Epicentre Technologies, Madison, WI, USA), 1.4 nM of each primer, and 2.5 units of Taq I DNA polymerase (Promega, Madison, WI, USA). Samples were amplified in an Eppendorf Thermocycler (Brinkmann Instruments, Westbury, NY, USA) under the following conditions: denaturing step at 95 ºC for 40 seconds; annealing step at 60 ºC for 30 sec; amplification step at 72 ºC for 1 min for 20 to 35cycles.

4. Production of a Parkinsonian rat model, and cell transplantation

Male Sprague-Dawley rats weighing 200-230g were used to generate Parkinsonian rat models. The surgical procedure was performed as described previously.[37,38]

In brief, animals per groups were housed in a temperature-controlled room on a 12hr.-light/12hr.-dark schedule with free access to food and water. Rats were anesthetized with a mixture of ketamine (75 mg/kg), acepromazine (0.75 mg/kg) and rompun (4 mg/kg) and mounted in a stereotaxic apparatus. A neurotoxin 6-OHDA hydrobromide (Sigma, St Louis, MO, 8 μg free base in 4 μl of 0.2 % ascorbic acid) was injected into the medial forebrain bundle

according to the following stereotaxic coordinates: AP –4.4 mm, ML 1.2 mm relative to bregma, and DV -7.5 mm from the dura. The injection was made at a rate of 0.5µl/min using a cannula, and was controlled using a Hamilton microsyringe. The connection between the cannula and the microsyringe was composed of polyethylene tubing. To prevent the noradrenergic neurons being destroyed, desipramine (12.5 mg/kg, i.p.) was administered 30 min prior to the 6-OHDA infusion.

After two weeks of the development of 6-OHDA-induced lesions, animals were tested for turning and forepaw adjusting stepping, as described in a previous study.[39] One week after behavioral testing, mouse ES cells were transplanted using a sterilized stainless steel needle (0.3 mm O.D.) connected to a Hamilton microsyringe. Undifferentiated mouse ESCs (the wild type D3 and Nurr1-expressing N2) were maintained and differentiated as described previously.[36] Naïve D3 and N2 –derived cells were transplanted into rat striatum at *in vitro* differentiation day 9 (precursor cells) of total 14 days. 4 $\mu\ell$ of the cell suspension (1×10^5 cells/$\mu\ell$) was injected into the ipsilateral striatum [AP, +0.2 mm; ML, 3.0mm; DV, 4.5mm (2 $\mu\ell$) and 5.5mm (2 $\mu\ell$), respectively] over a period of 3 min. A time lapse of 4 min before the removal of the needle allowed the cells to settle down. The rats were given an injection of cyclosporin A (10 mg/kg, i.p.; Chong Kun Dang Pharm., Seoul, Korea) 24 hr before grafting and this was continued until sacrifice. Behavioral testing of mouse ES cell grafted group was conducted after the 2nd, 4th, 6th, and 8th weeks of transplantation.

Experimental groups were divided into 4 groups: (ⅰ) a normal group, 10 rats without lesions; (ⅱ) a sham control group with 6-OHDA lesions (n=10); (ⅲ) a 6-OHDA lesioned group transplanted with neurons derived from wild-type mouse ES cells (D3, n=25); and (ⅳ) a 6-OHDA lesioned group transplanted

with neurons derived from Nurr-expressing ESCs (N2, n=25). Teratoma formation was observed in four animals among 50 animals for D3 and N2 cell grafts.

5. Behavioral Tests

After two weeks of the development of 6-OHDA-induced lesions, animals were tested for turning behavior (apomorphine at 0.1 mg/kg i.p., in saline containing ascorbic acid at 2 mg/ml; Sigma, St. Louis, MO, USA) and forepaw adjusting stepping, as described in a previous study.[39]

A. Forepaw adjusting step test

Two weeks after the development of 6-OHDA-induced lesions, the animals were moved across a table at a speed of 90 cm/12sec. Rats were held at the rear part of the torso by one hand with their hindlimbs lifted and one forepaw was held steady with another hand of the experimenter so as to bear weight on the other forepaw. During this interval, the number of adjusting steps of the weight-bearing forepaw to compensate for the movement of the body were counted. The speed of the belt on the treadmill was controlled by a d.c. servomotor (Bison Gear and Engineering Corp.) and a power controller unit (Motor Master, Glendale, CA). The belt of the treadmill was made of flexible rubber and the surface was covered with cloth tape to give a textured surface for the forepaw movements.

Each stepping test consisted of five trials for each forepaw, alternating between forepaws. In all experiments, the average of the five trials for each forepaw was used for analysis.

B. Apomorphine-induced rotation test

Drug-induced rotations were measured using an automated rotometer consisting of a rotation bowl and a tether attached to the torso of the rat. In 6-OHDA lesion experiments, apomorphine-induced rotation (0.1 mg/kg, i.p.) was determined two weeks after the 6-OHDA lesion, immediately following the stepping test. In all experiments, the total number of rotations during the 1 h test was used for analysis.

6. Extracellular single-unit microrecording

The extracellular unit recording procedure (normal rat: n=5, PD model: n=5, D3 graft: n=10, N2 graft: n=10) and statistical analysis were performed as described previously.[37] In brief, for the extracellular unit recordings, at 10 weeks after transplantation, the rats were anesthetized with urethane (1.3 mg/kg i.p.). A Glass microelectrode (WPI, Sarasota, FL, USA, impedance 7-10 Mohm at 100Hz) filled with 2.5% pontamine sky blue in 0.5 M sodium acetate buffer (pH 7.6) was used for single recordings. Microelectrodes were stereotaxically guided through the drilled skull burr hole to the target coordinates; (pedunculopontine nucleus(PPN): AP-7.8 mm, ML; 1.8 mm and DV; 6.6-7.2 mm; substantia nigra pars reticulata (SNpr): AP-5.3 mm, ML; 2.4 mm and DV; 7.4-8.0 mm; subthalamic nucleus (STN): AP-3.7 mm, ML; 2.5 mm and DV; 7.4-8.0 mm from the dura). Electrical signals were amplified using a DAM 80 preamplifier (WPI, Sarasota, FL, USA) in bridge mode. The signals was displayed on a storage oscilloscope and monitored with an audio amplifier (WPI, Sarasota, FL, USA). Single unit activity was isolated with a window discriminator, and firing rate data was collected on a computer equipped with Spike 2 software (version 2.18, Cambridge Electronic Design, UK). Visual inspection of digital neuronal activity and raster displays were

useful complements for the computer based analysis of the discharge patterns of the units. The isolated units were monitored for at least 10 min to ensure the stability of their firing rate, firing pattern and spike morphology, and then 5-10 min of spontaneous activity was recorded. At the end of recording, the location of the tip of the recording microelectrode was marked, at -15㎂ for 20-30 min, by an iontophoretic deposit of pontamine sky blue. After the completion of the recordings, animals were deeply anesthetized; their brain was perfused, removed, and sectioned for histological confirmation of the recording site. The storage signal was converted to square wave pulses with the aid of a window discriminator and a personal computer. The mean firing rate, the mean interspike interval (ISI) and discharge pattern were investigated for each neuron. The ISIs allowed an evaluation of the degrees of neuron's burst frequency, following an algorithm described by Hutchison et al.[40]

7. Histological assessment for transplanted TH^+ immunoreactive cells

Histological assessment for transplanted TH^+ cells (normal rat: n=4, PD model: n=5, D3 graft: n=16, N2 graft: n=17) was performed by modifying the methods reported previously.[25,39,41] Briefly, 10 weeks after transplantation of D3 and N2 cells, the grafted rats were anesthetized with 25% urethane (Sigma) in PBS and intracardially perfused with 125 ml of normal saline followed by 250 ml of ice-cold 4% paraformaldehyde in PBS. Brains were postfixed in the same solution, cryoprotected with 30% sucrose in PBS for 48 hours and frozen. Brains were sectioned on a freezing microtome (section thickness: 10㎛) and collected in PBS. Serial sections spanning the graft were

made. The staining of TH^+ neurons was performed using rabbit anti-TH antibody (Pel-Freeze Rogers, AK, USA, 1:250). Counting of TH^+ neurons was performed on every tenth of serial sections using a Zeiss Axioplan light microscope with a 20x lens. Quantitative data from serial sections was expressed as means±S.E.M/section.

8. Analysis of dopamine

For DA detection *in vivo*, animals in each group (normal rat: n=3, PD model: n=3, D3 graft: n=4, N2 graft: n=4) were implanted with microdialysis probes (CMA/11 Guide Cannula, CMA Microdialysis AB, Solna, Sweden) in the ipsilateral striatum (coordinates: AP to bregma, +1.2mm; LA from midline, 2.5mm; ventral to the surface of the dura, -6.0mm). The probe was perfused with artificial cerebrospinal fluid (aCSF) at a flow rate of 2㎕/min for 4 h (CMA102 Microdialysis Pump). The aCSF consisted of 145 mM, 2.7 mM KCl, 1.2 mM $CaCl_2 \cdot 2H_2O$, 1.0 mM $MgCl_2 \cdot 6H_2O$, 2.0 mM Na_2HPO_4, pH 7.4 with H_3PO_4. The Guide cell (+400 mV) was used to perform a partial clean-up of the mobile phase prior to sample injection. The samples were introduced into the autosampler at a mobile phase flow rate of 1.0 ml/min. Neurotransmitter separation was carried out by means of a reverse-phase column (ESA HR-80 column: 3㎛, ODS, 80X4.6 mm; ESA Bioscience, Cheimsford, MA, USA). Mobil phase consisted of 75 mM sodium phosphate monobasic, 1.7 mM OSA, Triethylamine, 0.1 mM EDTA, Acetonitrile.

Typical values of applied potential used in the present experiment were -100 mV at Electrode 1 and +200 mV at Electrode 2. Chromatograms were analyzed with peak areas, which were classified with the retention times of reference substances.

Ⅲ. RESULTS

1. High yield of DA neurons from mouse ES cells efficiently induces behavior recovery in a Parkinsonian rat model

The co-culturing of Nurr1-expressing mouse ESCs with PA6 stromal cells synergistically generate a high proportion of DA neurons up to 90% of total neurons, while naïve D3 cells induce a composition of about 28% DA neurons.[36] (Figure 1) These neurons expressed high levels of midbrain DA markers and released DA.

Nurr1 and SDIA may cooperatively induce the DA phenotype. Furthermore, when treated with the signaling molecules (SM), a much greater effect was observed with statistical significance ($p < .05$), and approximately 90% of $Tuj1^+$ neurons were TH^+ under the optical condition. We did not test the effect of individual signaling molecule in this experiment. However, the previous report indicates that each signaling molecule has a marginal effect on the differentiation into DA neurons and a much greater effect when treated in combination. [20]

TH^+ cells exist in many neuronal phenotypes including DA and noradrenergic (NA) neurons. Therefore, previous study was determined whether TH^+ neurons generated by the optimized *in vitro* condition II have the midbrain DA neuron phenotype. The *in vitro* differentiated N2 cells under condition II were co-stained using antibodies against TH, AADC, DAT, GABA and 5-HT.[36] (data not shown)

In order to investigate mRNA expression of midbrain DA neuron-specific markers, semiquantitative reverse transcription analysis was carried out for

N2 clones at day 0, 9, and 14 during in vitro differentiation of N2 ES cells after on the PA6 feeder layer.(Figure 2) Expression of TH mRNA was increased in N2+SM (II) from 9day differentiation. Additional DA markers expression of such as DAT, and sox1 were also dramatically upregulated on 14day differentiation of N2 ES cells in N2+SM(II) condition.

The extent and location of the lesions induced by the 6-OHDA were confirmed by assessing the loss of TH-immunoreactive cells and fibers in the substantia nigra pars compacta (SNc) and striatum in a rat Parkinsonian model with 6-OHDA. (Figure 3)

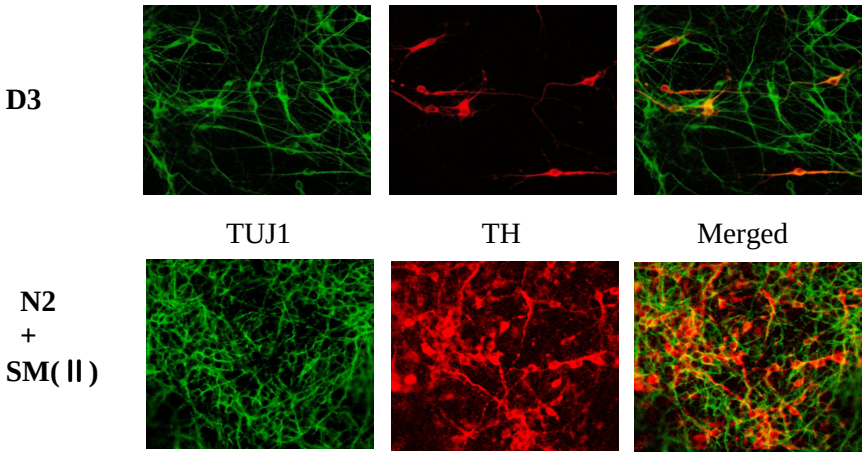

Figure 1. Immunostaining of TH^+ neurons generated by N2 cells under condition II. As a results of detection the β-tubulin and TH-positive cells by immunocytochemistry in differentiated D3 and N2 [N2+SM (II)] cells, the co-culturing of Nurr1-expressing mouse ESCs with PA6 stromal cells synergistically generate a high proportion of DA neurons compare to naïve D3 cells.

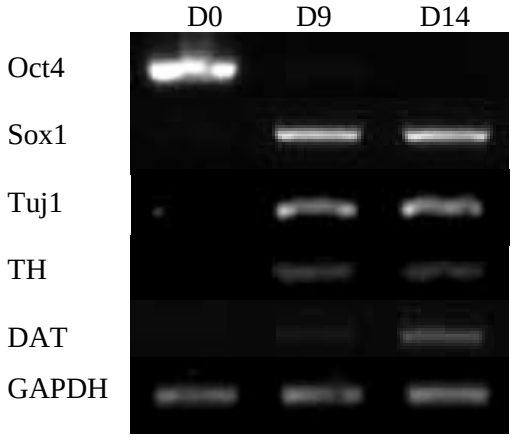

Figure 2. Semiquantitative reverse transcription polymerase chain reaction analysis for DA neuron markers.

Semiquantitative RT-PCR analyses of midbrain DA markers after *in vitro* differentiation of N2+SM(II) conditions. Expression of TH mRNA was increased in N2+SM (II) from 9day differentiation. Additional DA markers expression of such as DAT, and sox1 were also dramatically upregulated on 14day differentiation of N2 ES cells under the N2+SM(II) condition.

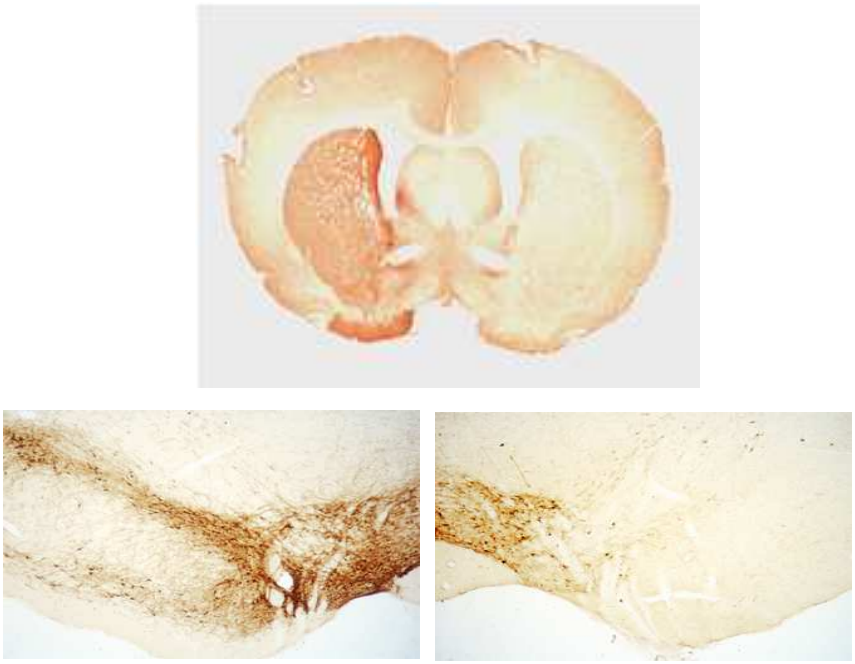

Figure 3. Immunohistochemistry of tyrosine hydroxylase (TH) in 6-OHDA treated side showing the total degeneration of dopamine fibers in the striatum(upper), and dopamine cell bodies in the SNc on the 6-OHDA injected side (lower, right) in hemiparkinsonian rat models compared to the normal side (left). SNpc: substantia nigra pars compacta

To examine the *in vivo* effect of the ESCs-derived DA neurons, the cells were transplanted into an animal model of PD. The PD models induced by the 6-OHDA were confirmed by the observed increase in the apomorphine-induced rotation(>500 turns/1hr.) and the observed reduction in the forepaw stepping number (Pre-TP in Table1 and 2). The extent and location of the lesions were also confirmed by assessing the loss of TH-immunoreactive cells and fibers in the SNpc and striatum.(Figure 3) After 3 weeks of lesion development, animals were given a suspension of $4x10^5$ ESCs into the striatum of PD models.

We transplanted the 4×10^5 cells into the striatum of rat PD models on the 9^{th} day of a 14 day *in vitro* differentiation. Effects of the grafted cells on behavior recovery were evaluated at the 2^{nd}, 4^{th}, 6^{th}, and 8^{th} weeks of transplantation with forepaw adjusting step (Figure 4) and an apomorphine-induced rotation test (Figure 5)

The forepaw adjusting step is a non-pharmacological test and thus reflects a more direct measure of motor deficits.[39] As shown in figure 4, a control PD group (sham) showed almost no stepping until 8 weeks ($0.08\pm0.08 \rightarrow 0.63\pm0.275$). However, the PD animals grafted with differentiated N2 cells (90% DA neurons/total neurons) showed a significant improvement over time in stepping number ($0.54\pm0.14 \rightarrow 10.49\pm0.64$) and recovery of >75% (>10 steps) at 8 weeks after transplantation as compared with the wild-type (~13 steps, data not shown). In contrast, the PD models grafted with naïve D3-derived cells (28% DA neurons/total neurons) showed a modest improvement in stepping over time, about 3 steps at 8 weeks postgrafting.

In 6-OHDA lesion experiments, apomorphine-induced rotation (0.1 mg/kg, i.p.) was determined two weeks after the 6-OHDA lesion, immediately following the stepping test. In all experiments, the total number of rotations

during the 1 hour test was used for analysis by apomorphine injection (Table 3, Figure 5).

The apomorphine stimulation of the PD models induces a movement bias contralateral to the lesion side. Sham controls showed a consistently high number of rotations (> 500), whereas the PD models grafted with N2-derived cells showed a significant reduction (496.6±33.22→60.6±24.2, at 8week) in rotational scores from 2 weeks posttransplantation compared with a sham control group (512.2±87.0→545±105.6, at 8 week). As in a forepaw adjusting step test (Figure 4), however, animals grafted with D3-derived cells showed a slight, but not significant, decrease in rotation compared with the PD animal grafted N2-derived cells. Graft sizes after transplantation of D3- and N2-derived cells were similar (data not shown). Taken together, high levels of DA neurons differentiated from N2 ESCs more efficiently bring about behavior recovery in a Parkinsonian rat model than did wild-type D3 ESC-derived neurons.

Table 1. The forepaw adjusting step[1] test after grafting ESCs in rat PD models.

Experimental Group[2]	Pre TP[3]	2	4	6	8
Sham	0.08±0.08	0.511±0.1	0.55±0.4	0.65±0.09	0.63±0.28
D3	0.51±0.17	1.53±0.47	2.1±0.62	2.37±0.512	3.03±1.53
N2	0.54±0.14	6.34±0.11**	8.23±1.21**	9.11±1.26**	10.49±0.64**

[1]. The results in the adjusting step test are expressed as the number of steps/0.9 m of treadmill (at a rate of 0.075 m/sec) with the lesioned side forepaw

[2]. Experomental groups were divided sham; a sham control group with 6-OHDA lesions, D3; a 6-OHDA lesioned group transplanted with neurons derived from wild-type mouse ES cells, and N2; a 6-OHDA lesioned group transplanted with neurons derived from Nurr1-expressing mouse ESCs

[3]. pre-TP; before transplantation, 2nd, 4th, 6th, and 8th weeks of transplantation.

* Significantly different from sham controls at $p<0.05$, ** at $p<0.01$.

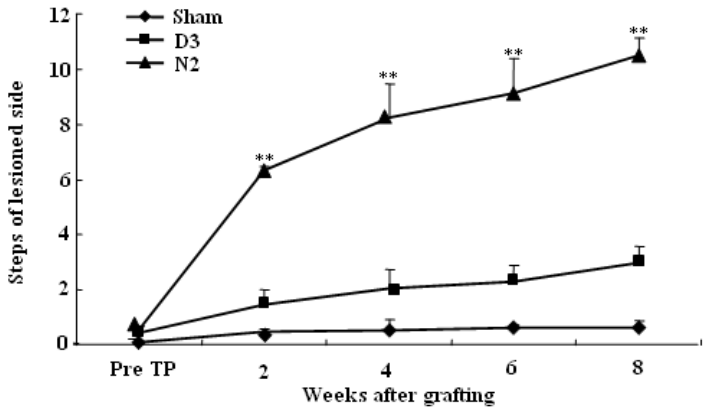

Figure 4. The forepaw adjusting stepping test after grafting mouse ESCs in rat PD models.

The behavior in PD animals grafted with neurons derived from wild-type D3 cells (n=25) or Nurr1-overexpressing N2 cells (n=25) and sham controls (n=10) was tested before transplantation (pre TP) and at 2, 4, 6, and 8 weeks postgrafting. A control PD group showed almost no stepping until 8 weeks. However, the PD animals grafted with differentiated N2 cells showed a significant improvement over time in stepping number at 8 weeks after transplantation as compared with the wild-type. The results in the adjusting step test are expressed as the number of steps/0.9 m of treadmill (at a rate of 0.075 m/sec) with the lesioned side forepaw.

Table 2. The apomorphine-induced rotation[1] test after grafting mouse ESCs in rat PD models.

Experimental Group[2]	Pre TP[3]	2	4	6	8
Sham	512.2±87.0	580.7±250	595.5±238	535.5±107	545±105.6
D3	580.7±86.2	499.2±121	483±121.6	425.2±65.4	374±85.1
N2	469.6±33.22	68.1±25.09**	136.5±33.9**	81.6±33.9**	60.6±24.2**

[1]. The results in the apomorphine-induced rotation (0.1 mg/kg, i.p.) test are expressed as the number of rotation/1hour after the 6-OHDA lesion.

[2]. Experomental groups were divided sham; a sham control group with 6-OHDA lesions, D3;a 6-OHDA lesioned group transplanted with neurons derived from wild-type mouse ES cells, and N2;a 6-OHDA lesioned group transplanted with neurons derived from Nurr1-expressing mouse ES cells

[3]. pre-TP; before transplantation, 2nd, 4th, 6th, and 8th weeks of transplantation.

* Significantly different from sham controls at $p<0.05$, ** at $p<0.01$.

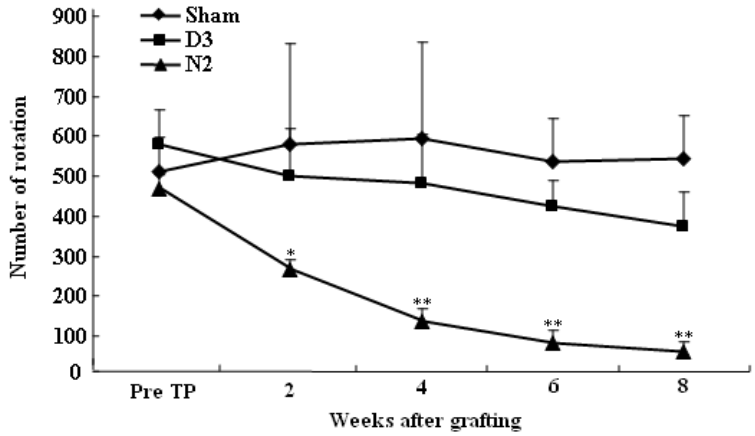

Figure 5. The apomorphine-induced rotation test after grafting mouse ESCs in rat PD models.

The behavior in PD animals grafted with neurons derived from wild-type D3 cells (n=25) or Nurr1-overexpressing N2 cells (n=25) and sham controls (n=10) was tested before transplantation (pre TP) and at 2, 4, 6, and 8 weeks postgrafting. Apomorphine-induced rotation response per hour. Animals with sham surgery showed no change in rotational score over time. In contrast, animals with N2 cells showed a significant reduction in rotation over time. Animals with D3 cells showed modest, but non-significant reduction. * Significantly different from sham controls at $p<0.05$, ** at $p<0.01$.

2. Electrophysiological effects of grafted mouse ES cells

To electrophysiologically investigate the transplantation effect, we examined the firing rates and firing patterns in the PPN, SNpr, and STN by extracellular single-unit microelectrode recording. In PD, the progressive deficit of DA cells in the SNpc results in hyperactivity in the SNpr, STN, and PPN due to impaired information processing in the basal ganglia.[3,5,6] As previously reported,[37,42,43] we observed hyperactivity in the SNpr, STN, and PPN of 6-OHDA lesioned rats. (Table 3) PD models with sham surgery exhibited a significant increase in the mean firing rates of three areas compared to normal rats. (PPN; 16.05±2.8→21.87±2.3, STN; 21.23±2.0→34.28±3.8, SNpr; 23.02±1.75→20.05±2.14)

Following ES cell grafting in the PD models, however, the mean firing rates were significantly decreased in all three areas compared with sham controls 10 weeks postgrafting (Figure 6). The firing rates in PPN and SNpr were slightly lower in N2 grafts than in D3 grafts, but the firing rate in STN was almost the same between the two grafts. However, the reason the firing rates in the grafts became lower than those in normal rats is yet to be elucidated. Representative firing patterns in the SNpr of each group are shown in figure 7. Neuronal activity of a sham control was higher (27.8 spikes/sec) compared to that in a normal rat (20.8 spikes/sec). In hemi-Parkinsonian models grafted with DA neurons derived from N2 cells, the firing activity was significantly decreased (10.4 spikes/sec).

These results demonstrate that reinnervation of DA neurons by the grafting of ES cells induces decreased firing rates in SNpr, STN, and PPN neurons. Each position of extracellular single-unit microelectrode recording was confirmed by the identification of neurons by their stereotaxic location and the histological location of the electrode tip after iontophoresis with pontamine

sky blue (-18 μA for 20 min) (Figure 8) by staining with cresyl-violet staining. We confirmed position of SNpr recording stained with cresyl violet, and compared to rat atlas. [38]

Table 3. *In vivo* electrophysiological effects[1] of grafted ES cells in rat PD models.

Experimental Group[2]	PPN[3]	STN[4]	SNpr[5]
Normal	16.05±2.8	21.23±2.0	23.02±1.75
Sham	21.87±2.3	34.28±3.8	30.05±2.14
D3	12.78±1.2**	10.35±1.22**	16.14±2.04*
N2	10.62±0.9**	10.79±1.43**	9.00±0.95**

[1]. The extracellular single-unit recordings were performed from STN, SNpr, and PPN in hemi-Parkinsonian models grafted with DA neurons derived from D3 or N2 cells and in sham controls. The results in electrophysiological recording were expresses mean firing rate.

[2]. Experomental groups were divided sham; a sham control group with 6-OHDA lesions, D3;a 6-OHDA lesioned group transplanted with neurons derived from wild-type mouse ES cells, and N2;a 6-OHDA lesioned group transplanted with neurons derived from Nurr1-expressing mouse ES cells

[3]. PPN; pedunculopontine nucleus

[4]. STN; subthalamic nucleus

[5]. SNpr; substantia nigra pars reticulata

* Significantly different from sham controls at $p<0.05$, ** at $p<0.01$.

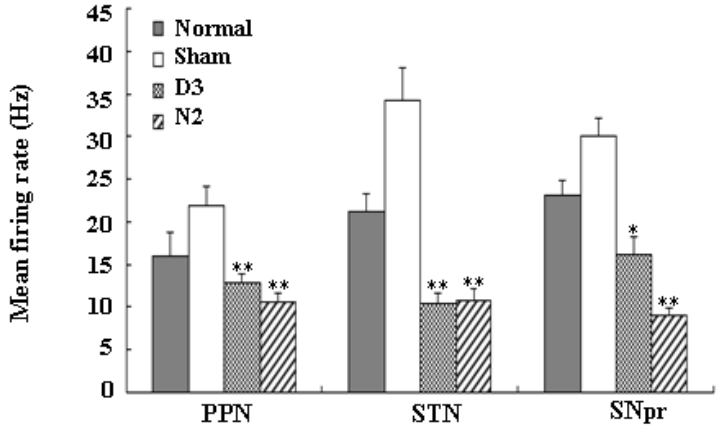

Figure 6. *In vivo* electrophysiological effects of grafted ESCs in PD rat.
The extracellular single-unit recordings were performed from STN, SNpr, and PPN in hemi-Parkinsonian models grafted with DA neurons derived from D3 or N2 cells and in sham controls at 10 weeks postgrafting. Compared to the normal rats, PD rat models exhibited a significant increase in mean firing rates in the SNpr, STN, and PPN. Following ES cell (D3 or N2) grafting in the PD models, the mean firing rates in the SNpr, STN, and PPN were significantly decreased. *$p<0.05$, **$p<0.01$ in comparision with values from sham controls. PPN; pedunculopontine nucleus, STN; subthalamic nucleus, SNpr; substantia nigra pars reticulata

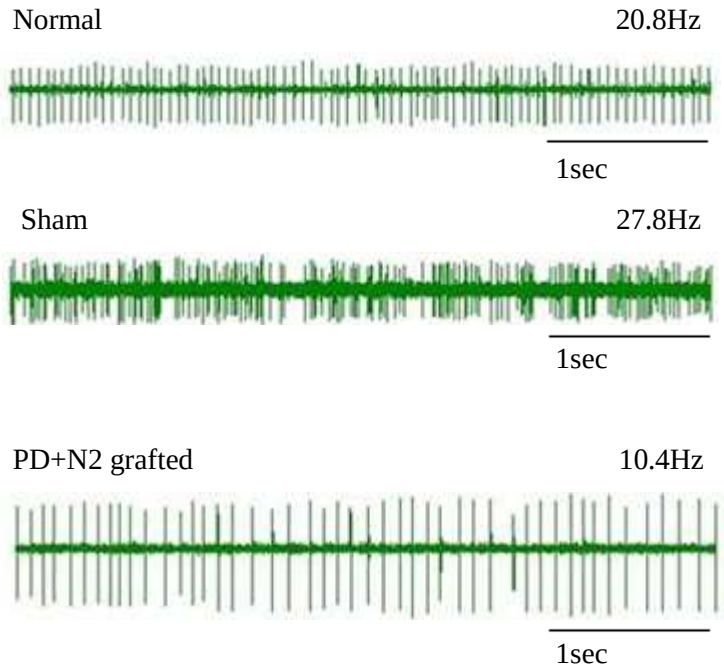

Figure 7. Representative discharge patterns of SNpr recorded in each group.

Neuronal activity of a sham control was higher (27.8 spikes/sec) compared to that in a normal rat (20.8 spikes/sec). In hemi-Parkinsonian models grafted with DA neurons derived from N2 cells, the firing activity was significantly decreased (10.4 spikes/sec). PPN; pedunculopontine nucleus, STN; subthalamic nucleus, SNpr; substantia nigra pars reticulata

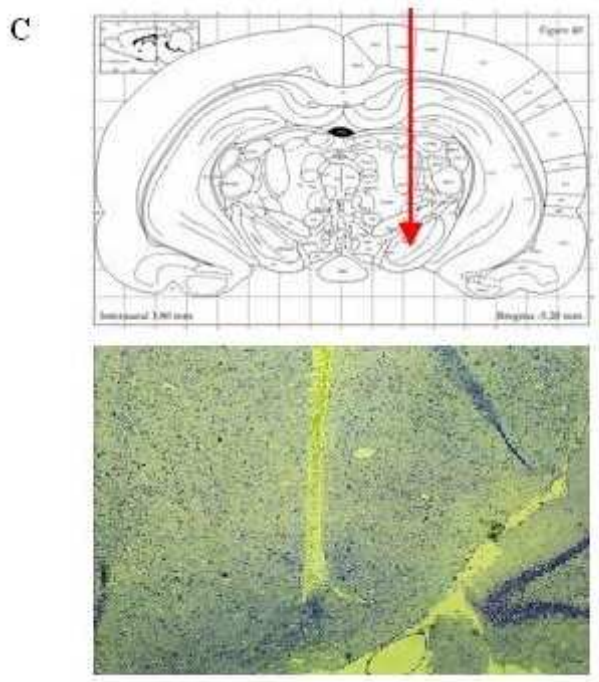

Figure 8. Cresyl-violet staining of recording site in SNpr

Cresyl-violet staining showing the pontamine sky blue mark corresponding to a neuron which was recorded at the end of a track in SNpr. Each position of extracellular single-unit microelectrode recording was confirmed by the identification of neurons by their stereotaxic location and the histological location of the electrode tip after iontophoresis with pontamine sky blue (-18 μA for 20 min) Magnification, 40X. SNpr; substantia nigra pars reticulata

3. DA production of grafted ES cells

To examine the correlation between the behavior recovery and the number of DA neurons in the grafts, we next analyzed the TH^+ cells which originated from ESCs in the striatum of the rat brain (Figures 9, 10). Ten weeks after transplantation, the recipient rats were sacrificed, fixed by perfusion, and then analyzed for TH^+ neurons by immunostaining. In the N2-derived cell grafts, we were able to find many TH^+ cell bodies (Figure 9, right). In contrast, fewer TH^+ cell bodies were detected in the D3-derived cell grafts (Figure 9, left), although the same number of cells was transplanted in both cases. When we counted the number of TH^+ cells (Figure 10), we determined the TH^+ cells/section to be 187.63±18.7 for the N2 graft and 52.13±5.33 for the D3 graft.

The difference in the number of TH^+ cells is consistent with the difference in behavior recovery between N2 and D3 grafts, indicating a good correlation between TH^+ cell numbers and behavior recovery.

For DA detection *in vivo*, animals in each group were implanted with microdialysis probes in the ipsilateral striatum. The probe was perfused with aCSF at a flow rate of 2$\mu\ell$/min for 4 hour, collected the sample and then analyzed of dopamine by reverse-phase HPLC.(Figure 11). The DA level in PD models was much lower than that in normal rats, whereas the DA level (~0.08 μM) in animals grafted with N2-derived cells was almost the same as that (~0.09 μM) of normal rats (Figure 12), showing that transplanted N2 cells efficiently release DA, and thus, induce functional recovery of brain circuitry. These results have the correlation the behavior recovery and the histological assessments. However, DA level (~0.05 μM) secreted by the D3-derived cell grafts was lower than that (~0.08 μM) by N2 cell grafts. Taken

together, our results show that the high yield of DA neurons generated from mouse ES cells also efficiently functions *in vivo* after transplantation in a Parkinsonian rat model.

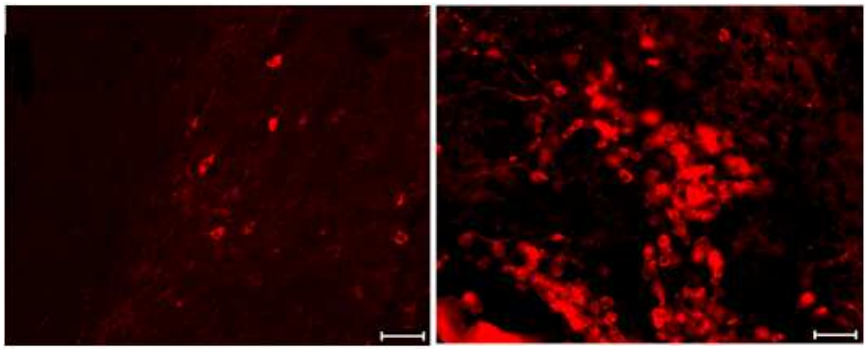

Figure 9. Survival of N2 cells after grafting mouse ESCs into the striatum of hemi-Parkinsonian rats.

Immunohistochemical staining using anti-TH antibody at 10 weeks after implantation of D3 and N2 ES cells into 6-OHDA lesioned striatum. Numerous TH^+ neurons were found within the N2 cells grafts (right) in comparison to D3 cell grafts (left). Scale bars, 50 ㎛.

D3;a 6-OHDA lesioned group transplanted with neurons derived from wild-type mouse ESCs, and N2;a 6-OHDA lesioned group transplanted with neurons derived from Nurr1-expressing mouse ESCs

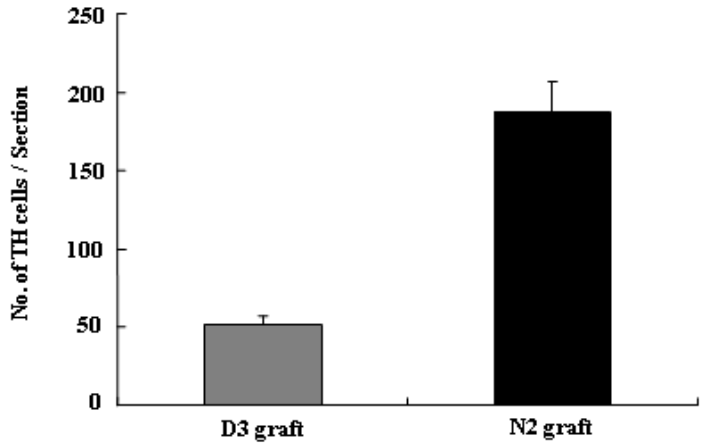

Figure 10. Number of TH^+ neurons/section in the striatum grafts.

Immunohistochemical staining using anti-TH antibody at 10 weeks after implantation of D3 and N2 ES cells into 6-OHDA lesioned striatum was analyzed by counting the number of DA neuron/section. Number of TH^+ neurons/section in the striatum after transplantation ($4x10^5$ cells) with D3 and N2 ESCs. The number of DA neurons/section in N2 and D3 grafts was 187.63±18.7 and 52.13±5.33, respectively. D3;a 6-OHDA lesioned group transplanted with neurons derived from wild-type mouse ES cells, and N2;a 6-OHDA lesioned group transplanted with neurons derived from Nurr1-expressing mouse ESCs

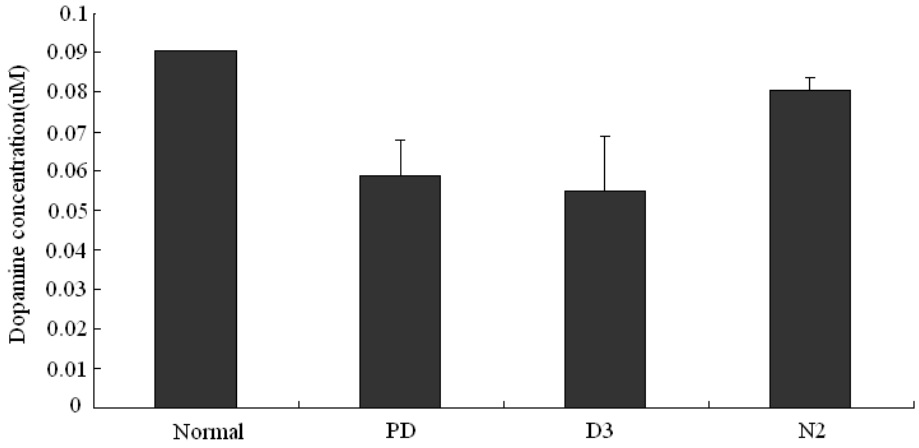

Figure 11. The production of DA in the striatum of grafted mouse ES cells. The amount of DA in the striatum was analyzed by microdialysis. DA level (~0.08 μM) secreted by the N2 ES cell graft, but not D3 cell graft (~0.05 μM), in a hemi-Parkinsonian striatum was similar to that (~0.09 μM) of a normal rat. D3;a 6-OHDA lesioned group transplanted with neurons derived from wild-type mouse ES cells, and N2;a 6-OHDA lesioned group transplanted with neurons derived from Nurr1-expressing mouse ESCs

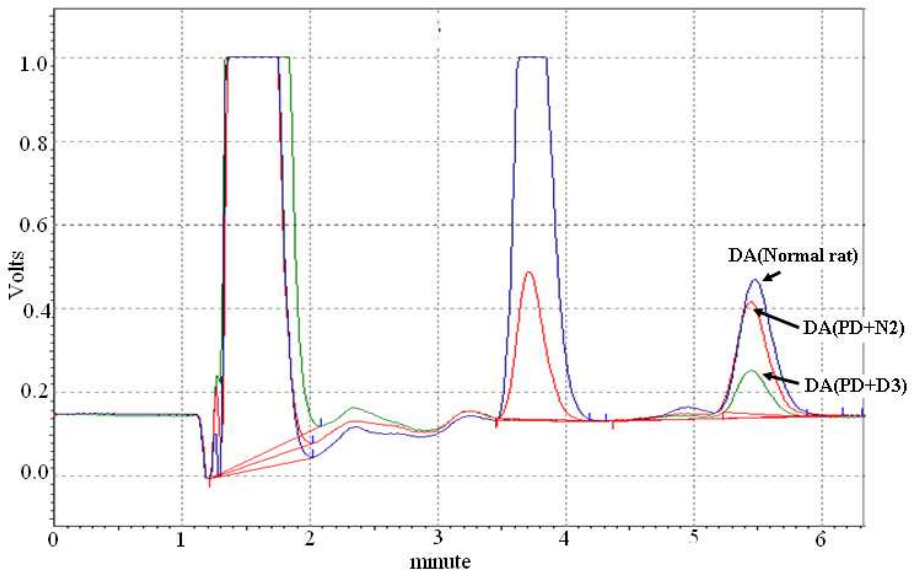

Figure 12. DA analysis in the striatum of grafted mouse ESCs.

The amount of DA in the striatum was analyzed by microdialysis. DA level (~0.08 μM) secreted by the N2 ES cell graft, but not D3 cell graft (~0.05 μM), in a hemi-Parkinsonian striatum was similar to that (~0.09 μM) of a normal rat.

Ⅳ. DISCUSSION

The distinguishing features of ESCs are their capacity to be maintained indefinitely in an undifferentiated state and the ability to develop into multilineage cells under certain conditions. By overexpressing the Nurr1 transcription factor (N2 cell line) and coculturing with PA6 stromal cells in the presence of signaling molecules, such as SHH and FGF8, we have developed an *in vitro* protocol by which a high yield of DA neurons (90% of total neurons) was efficiently generated from mouse ES cells (figure 1).[36] In this study, we attempted to transplant the DA neurons produced by this protocol into a Parkinsonian rat model to determine whether these neurons also efficiently bring about a functional improvement in PD animal models.

Behavior recovery by N2 cell grafting (~90% DA neurons/total neurons) was much more prominent in both apomorphine-induced rotation and forepaw stepping tests compared to naïve D3 cell grafting (~28% DA neurons/total neurons) (Figure 4,5).

In the forepaw stepping test, Animals grafted with N2 cells showed a dramatic increase (0.54±0.14→10.49±0.64 at 8 weeks) compared to sham controls (0.08±0.08→0.63±0.275 at 8 weeks). Animals with D3 cells showed modest, but non-significant increase. In another behavioral test, apomorphine-induced rotation test, animals with sham surgery showed no change in rotational score over time (545±105.6 at 8 weeks). In contrast, animals with N2 cells showed a significant reduction in rotation over time (60.64±24.2 at 8 weeks).

These results indicate that the efficient *in vitro* protocol can be translated to the *in vivo* context well. In the PD model, hyperactivity in the SNpr, STN, and PPN can be induced by impaired information processing in the basal

ganglia which results from the death of DA cells in the SNpc. A current model of the motor pathway postulates that D1 dopamine receptors on striatonigral neurons act to increase the activity of the inhibitory GABAergic striatonigral projection.[44,45] Thus, due to the DA loss of the striatum in rat PD models, striatal GABAergic neurons projecting to SNpr should become underactive, leading to less inhibition of this output nucleus. This, in turn, should lead to overactivation of the GABAergic SNpr neurons, which would be further amplified by an increased excitatory input from the STN.[46] The hyperactivity of the STN is based on the hypothesis that the loss of DA in the striatum causes a reduction in the activity of the inhibitory GABAergic pallidosubthalamic pathway.[3] Interestingly, hyperactivity in the SNpr, STN, and PPN of PD models was dramatically reduced by the transplantation of ES cell-derived neurons (Figure 6), which signifies the restoration of neuronal circuitry. In PPN and SNpr, the firing rates were slightly lower in N2 grafting than in D3 grafting, although they were similar in STN.

Figure 9 shows that, 10 weeks after transplantation, a higher number of DA neurons were detected in the striatum of N2-grafted animals compared with that of D3-grafted animals although graft sizes between two were similar. The number of DA neurons was 3.6 fold higher in N2 grafted animals than in D3 grafted animals (Figure 10). This difference in the number of DA neurons led to the difference in the behavior recovery of the N2- and D3-grafted animals (Figure 4, 5). Although the DA content in PD models with D3-derived cells was low (~0.05 μM), the DA content (~0.08 μM) secreted from PD animals with N2-derived cells was similar to that (~0.09 μM) of the normal rat (Figure 11), and the level of behavior recovery in the PD model with N2-derived cells nearly reached the level of normal rats by 8 weeks after grafting (Figure 4, 5). Thus, this DA secretion level correlates well with the degree of behavior recovery. Therefore, these results suggest that our *in vitro*

protocol is also very effective in the *in vivo* context after transplantation. The reinnervation of DA neurons by the transplantation of ES cell-derived neurons is believed to improve Parkinsonian motor symptoms, presumably by reducing the activity of the basal ganglia output structures.[39,47] The observed behavioral effects were dependent on the survival of DA neurons within the striatum, since grafting of other tissue produces no behavioral effects.[48,49] Following the development of an efficient differentiation method from mouse ES cells and application of these cells to a Parkinsonian rat model, in the next step, we are now pursuing the efficient induction of DA neurons from human ES cells and their application to Parkinsonian animal models.

Ⅴ. CONCLUSION

In this study, we attempted to transplant the DA neurons produced by overexpressing the Nurr1 transcription factor (N2 cell line) and coculturing with PA6 stromal cells in the presence of signaling molecules, such as SHH and FGF8 into a Parkinsonian rat model to determine whether these neurons also efficiently bring about a functional improvement in PD animal models.

The PD animals grafted with N2-derived cells showed significant behavior improvements. Furthermore, hyperactivity observed in PD rat models was dramatically reduced by the grafting of N2-derived cells. The number of DA neurons in the striatum which originated from N2 grafting was much higher compared to that from D3 grafting, and the neurons efficiently released DA in the brain, showing a good correlation with behavioral recovery.

The reinnervation of DA neurons by the transplantation of ESC-derived neurons is believed to improve Parkinsonian motor symptoms, presumably by reducing the activity of the basal ganglia output structures.

REFERENCES

1. Youdim MB, Riederer P, Understanding Parkinson's disease. Sci Am 1997;276:52-9.
2. Betarbet R, Sherer TB, Di Monte DA, Greenamyre JT. Mechanistic approaches to Parkinson's disease pathogenesis. Brain Pathol 2002;12:499-510.
3. Albin RL, Young AB, Penney JB. The functional anatomy of basal ganglia disorders. Trends Neurosci 1989;12:366-75.
4. Alexander GE, Crutcher MD, DeLong MR. Basal ganglia-thalamocortical circuits: parallel substrates for motor, oculomotor, "prefrontal" and "limbic" functions. Prog Brain Res 1990;85:119-46.
5. Delfs JM, Ciaramitaro VM, Parry TJ, Chesslet MF. Subthalamic nucleus lesions: widespread effects on changes in gene expression induced by nigrostriatal dopamine depletion in rats. J Neurosci 1995;15: 6562-75.
6. Aziz TZ, Peggs D, Sambrook MA, Crossman AR. Lesion of the subthalamic nucleus for the alleviation of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced parkinsonism in the primate. Mov Disord 1991;6:288-92.
7. Benazzouz A, Gross C, Feger J, Boraud T, Bioulac B. Reversal of rigidity and improvement in motor performance by subthalamic high-frequency stimulation in MPTP-treated monkeys. Eur J Neurosci 1993;5:382-9.
8. Benabid AL, Pollak P, Gross C, Hoffmann D, Benazzouz A, Gao DM, et al. Acute and long-term effects of subthalamic nucleus stimulation in Parkinson's disease. Stereotact Funct Neurosurgy 1994;62:76-84.
9. Limousin P, Pollak P, Benazzouz A, Hoffmann D, Le Bas JF, Broussolle E et al. Effect of parkinsonian signs and symptoms of bilateral

subthalamic nucleus stimulation. Lancet 1995;345:91-5.

10. Pollak P, Benabid AL, Limousin P, Benazzouz A, Hoffmann D, Le Bas JF et al. Subthalamic nucleus stimulation alleviates akinesia and rigidity in parkinsonian patients. Adv Neurol 1996;69:591-4.
11. Olanow CW, Obeso JA. Levodopa motor complications in Parkinson's disease. Ann Neurol 2000;47:167-78.
12. Freed CR, Greene PE, Breeze RE, Tsai WI, Du Mouchel W, Kao R et al. Transplantation of Embryonic Dopamine Neurons for Severe Parkinson's Disease. N Engl J Med 2001;344:710-9.
13. Hauser RA, Freeman TB, Snow BJ, Nauert M, Gauger L, Kordower JH et al. Long-term evaluation of bilateral fetal nigral transplantation in Parkinson's disease. Arch Neurol 1999;56:179-87.
14. Backlund EO, Granberg PO, Hamberger B, Knutsson E, Martensson A, Sedvall G et al. Transplantation of adrenal medullary tissue to striatum in parkinsonism. First clinical trials. J Neurosurg 1985;62:169-73.
15. Madrazo I, Leon V, Torres C, Aguillera MC, Varela G, Alvarez F et al. Transplantation of fetal substantia nigra and adrenal medulla to the caudate nucleus in two patients with Parkinson's disease. N Engl J Med 1988;318:51.
16. Lindvall O. Transplantation into the human brain: present status and future possibilities. J Neurol Neurosurg Psychiatry Suppl 1989:39-54.
17. Date I, Imaoka T, Miyoshi Y, Ono T, Asari A, Ohmoto T. Chromaffin cell survival and host dopaminergic fiber recovery in a patient with Parkinson's disease treated by cografts of adrenal medulla and pretransected peripheral nerve. J Neurosurg 1996;84:685-9.
18. Deacon T, Schumacher J, Dinsmore J, Thomas C, Palmer P, Kott S et al. Histological evidence of fetal pig neural cell survival after transplantation into a patient with Parkinson's disease. Nat Med 1997;3:350-3.

19. Okabe JS, Forsberg-Nilsson K, Spiro AC, Segal M, McKay RD. Development of neuronal precursor cells and functional postmitotic neurons from embryonic stem cells in vitro. Mech Dev 1996;59:89-102.
20. Lee SH, Lumelsky N, Studer L, Auerbach JM, McKay RD. Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. Nat Biotechnol 2000;18:675-9.
21. Kawasaki H, Mizuseki K, Nishikawa S, Kaneko S, Kuwana Y, Nakanishi S et al. Induction of midbrain dopaminergic neurons from ES cells by stromal cell-derived inducing activity. Neuron 2000;28:31-40.
22. Castillo SO, Baffi JS, Palkovits M, Goldstein DS, Kopin IJ, Witta J et al. Dopamine biosynthesis is selectively abolished in substantia nigra/ventral tegmental area but not in hypothalamic neurons in mice with targeted disruption of the Nurr1 gene. Mol Cell Neurosci 1998;11:36-46.
23. Saucedo-Cardenas O, Quintana-Hau JD, Le WD, Smidt MP, Cox JJ, De Mayo F et al. Nurr1 is essential for the induction of the dopaminergic phenotype and the survival of ventral mesencephalic late dopaminergic precursor neurons. Proc Natl Acad Sci USA 1998;95:4013-8.
24. Zetterstrom RH, Solomin L, Jansson L, Hoffer BJ, Olson L, Perlmann T. Dopamine neuron agenesis in Nurr1-deficient mice. Science 1997;276:248-50.
25. Kim JH, Auerbach JM, Rodriguez-Gomez JA, Velasco I, Gavin D, Lumelsky Let al. Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease. Nature 2002;418:50-6.
26. Chung S, Sonntag KC, Andersson T, Bjorklund LM, Park JJ, Kim DW et al. Genetic engineering of mouse embryonic stem cells by Nurr1 enhances differentiation and maturation into dopaminergic neurons. Eur J Neurosci 2002;16:1829-38.

27. Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci* USA. 1981;78:7634-8.
28. Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature 1981;292:154-6.
29. Smith AG. Mouse embryo stem cells: their identification, propagation and manipulation. Semin Cell Biol 1992;3:385-99.
30. Desbaillets I, Ziegler U, Groscurth P, Gassmann M. Embryoid bodies: an in vitro model of mouse embryogenesis. Exp Physiol 2000;85:645-51.
31. Kehat I. *et al*. Human embryonic stem cells can differentiate into myocytes with structure and functional properties of cardiomyocytes. J Clin Invest 2001;108:407-14.
32. Wichterle H, Lieberam I, Porter JA, Jessell TM. Directed differentiation of embryonic stem cells into motor neurons. Cell 2002;110:385-97.
33. Damier P, Hirsch EC, Agid Y. The substantia nigra of the human brain. II. Patterns of loss of dopamine-containing neurons in Parkinson's disease. Brain 1999;122:1437-48.
34. Olanow CW, Tatton WG.. Etiology and pathogenesis of Parkinson's disease. Annu Rev Neurosci 1999;22:123-144.
35. Takagi, Y. *et al*. Dopaminergic neurons generated from monkey embryonic stem cells function in a Parkinson primate model. J Clin Invest 2005;115:102-9.
36. Kim DW, Chung SM, Hwang MK, Ferree A, Tsai HC, Park JJ et al. Stromal cell-derived inducing activity, Nurr 1 and signaling molecules synergically induced dopaminergic neurons from mouse embryonic stem cells. Stem cells 2006;24:557-67.
37. Chang JW, Yang JS, Jeon MF, Lee BH, Chung SS. Effect of subthalamic lesion with kainic acid on the neuronal activities of the basal ganglia of

rat Parkinsonian models with 6-hydroxydopamine. Acta Neurochir 2003;87:163-8.

38. Paxinos G, Watson C, The rat brain in stereotaxic coordinates. San Diego: Academic Press 1998.
39. Chang JW, Wachtel SR, Young D, Kang UJ. Biochemical and anatomical characterization of forepaw adjusting steps in rat models of Parkinson's disease: studies on medial forebrain bundle and striatal lesions. Neuroscience 1999;88:617-28.
40. Hutchison WD, Allan RJ, Opitz H, Levy R., Dostrovsky JO, Lang AE et al. Neurophysiological identification of the subthalamic nucleus in surgery for Parkinson's disease. Ann Neurol 1998;44: 622-8.
41. Shim JW, Koh HC, Chang MY, Roh E, Choi CY, Oh YJ et al. Enhanced in vitro midbrain dopamine neuron differentiation, dopaminergic function, neurite outgrowth, and 1-methyl-4-phenylpyridium resistance in mouse embryonic stem cells overexpressing Bcl-XL. J Neurosci 2004;24:843-52.
42. Burbaud P, Gross C, Benazzouz A, Coussemacq M, Bioulac B. Reduction of apomorphine-induced rotational behaviour by subthalamic lesion in 6-OHDA lesioned rats is associated with a normalization of firing rate and discharge pattern of pars reticulata neurons. Exp Brain Res 1995;105:48-58.
43. Murer MG, Riquelme LA, Tseng KY, Cristal A, Santos J, Pazo JH. D1-D2 dopamine receptor interaction: an in vivo single unit electrophysiological study. Neuroreport 1997;8:783-7.
44. DeLong MR. Primate models of movement disorders of basal ganglia origin, Trends Neurosci 1990;13:281-5.
45. Hassani OK, Mouroux M, Feger J. Increased subthalamic neuronal activity after nigral dopaminergic lesion independent of disinhibition via the globus pallidus. Neuroscience 1996;72:105-15.

46. Limousin P, Krack P, Pollak P, Benazzouz A, Ardouin C, Hoffmann D et al. Electrical stimulation of the subthalamic nucleus in advanced Parkinson's disease. N Engl J Med 1998;339:1105-11.
47. Benazzouz A, Piallat B, Pollak P, Benabid AL. Responses of substantia nigra pars reticulata and globus pallidus complex to high frequency stimulation of the subthalamic nucleus in rats: electrophysiological data. Neurosci Lett 1995;189:77-80.
48. Dunnett SB, Hernandez TD, Summerfield A, Jones GH, Arbuthnott G. Graft-deriv[5]ed recovery from 6-OHDA lesions: specificity of ventral mesencephalic graft tissues. Exp Brain Res 1988;71:411-24.
49. Brownell AL, Livni E, Galpern W, Isacson O. In vivo PET imaging in rat of dopamine terminals reveals functional neural transplants. Ann Neurol 1998;43:387-90.

국문요약

6-하이드록시도파민에 의해 유도된 흰쥐 파킨슨병 모델에
쥐배아줄기세포에서 유래된 도파민신경세포의 이식 및 분석

(지도교수 장 진 우)

연세대학교 대학원 의과학과

조 윤 희

배아줄기세포는 선조체내의 특정 세포타입인 도파민 신경세포의 손실에 의해 발생되는 파킨슨씨 병에 또 하나의 치료방법으로 대두되고 있다. Nurr1유전자를 발현시킨 생쥐의 배아줄기세포에 PA6-stromal cell과 함께 배양하는 동안 이들이 도파민 신경세포로의 분화를 돕는 여러 인자들을 첨가하여 배양한 세포 N2로부터 유래된 도파민 신경세포 (전체 신경세포의 90%)를 흰쥐 파킨슨씨병 동물모델에 이식을 하여 그 효과를 분석하였다. N2세포에서 유래된

신경세포를 이식 받은 파킨슨씨 동물모델은 이식 2주부터 대조 실험동물군과 비교하여 행동학적인 향상이 나타났다. 반면 배양 시 아무런 처리를 하지 않은 배아줄기 세포인 D3세포 (전체 신경세포의 약 28%)를 이식 받은 동물군에서는 약간의 회복은 보였지만 유의미한 결과를 얻지는 못하였다. 또한 흰쥐 파킨슨씨병 동물모델의 시상밑핵, 대뇌각교뇌핵, 흑색질의 그물부분에서 과도한 출력에 대해 관찰한 결과, N2 세포에서 유래된 신경세포를 이식 받은 실험동물 군에서 과도한 출력이 유의미하게 감소되는 것을 확인 할 수 있었다. 유전자 조작된 N2세포를 이식 받은 실험 동물군의 선조체에서 유전자 조작을 받지 않은 D3세포를 이식한 실험 동물군에 비해 많은 수의 도파민 신경세포가 발견되었고, 이것은 N2세포를 이식 받은 실험동물군의 뇌에서 효율적으로 분비되는 도파민의 양과 행동상의 향상과도 일치하는 결과를 얻었다.

핵심이 되는 말 ; 배아줄기세포, 분화, 도파민 신경 세포, 파킨슨씨 병, 행동학적 회복

Publication List

Cho YH, Kim DA, Kim P, Hwang Y, Cho MS, Moon SY, Kim DW, Chang JW. Dopamine neurons derived from embryonic stem cells efficiently induced behavioral recovery in a Parkinsonian rat model. BBRC 2006;341:6-12