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Article

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Dopaminergic neurons generated from monkey embryonic stem cells function in a Parkinson primate model

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Parkinson disease (PD) is a neurodegenerative disorder characterized by loss of midbrain dopaminergic (DA) neurons. ES cells are currently the most promising donor cell source for cell-replacement therapy in PD. We previously described a strong neuralizing activity present on the surface of stromal cells, named stromal cell–derived inducing activity (SDIA). In this study, we generated neurospheres composed of neural progenitors from monkey ES cells, which are capable of producing large numbers of DA neurons. We demonstrated that FGF20, preferentially expressed in the substantia nigra, acts synergistically with FGF2 to increase the number of DA neurons in ES cell–derived neurospheres. We also analyzed the effect of transplantation of DA neurons generated from monkey ES cells into 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine–treated (MPTP-treated) monkeys, a primate model for PD. Behavioral studies and functional imaging revealed that the transplanted cells functioned as DA neurons and attenuated MPTP-induced neurological symptoms.

Introduction

Parkinson disease (PD) is a neurodegenerative disorder characterized by the loss of midbrain dopaminergic (DA) neurons, with subsequent reductions in striatal dopamine levels. While initial pharmacological treatment with L-dihydroxyphenylalanin (L-DOPA) can attenuate symptoms, the efficacy of this treatment gradually decreases over time. The development of motor complications then requires additional treatments, including deep brain stimulation and fetal DA neuron transplantation (1–3). Both studies of animal models and clinical investigations have shown that transplantation of fetal DA neurons can produce symptomatic relief (4–8). The technical and ethical difficulties in obtaining sufficient and appropriate donor fetal brain tissue, however, have limited the application of this therapy.

ES cells are self-renewing, pluripotent cells derived from the inner cell mass of the preimplantation blastocyst. These cells have many of the characteristics required of a cell source for cell-replacement therapy, including proliferation and differentiation capacities (9). We previously discovered that a strong neuralizing activity, which we called stromal cell–derived inducing activity (SDIA), is present

on the surface of stromal cells. In the absence of exogenous bone morphogenic protein–4, mouse ES cells differentiate efficiently into neural precursors and neurons when cultured for 1 week on SDIA-expressing mouse stromal cells (PA6 cells) (10). Recently, SDIA induction has also been applied to primate ES cells, which generated large numbers of neural precursors and postmitotic neurons when cultured on PA6 cells for two weeks (11). The SDIA method is both technically simple and efficient, producing significant numbers of midbrain DA neurons (10, 11).

Self-renewing, multipotent neural progenitors can be cultured as neurospheres (12). In this study, we generated neural progenitors from monkey ES cells, then expanded them as neurospheres, which contained progenitors of DA neurons. In addition, we analyzed the effect of FGF20, a novel member of the FGF family of growth factors that is expressed exclusively in the substantia nigra of the brain and is reported to have a protective effect on DA neurons (13). We observed increased DA neuron induction following treatment with FGF20. Furthermore, we transplanted neurons generated by this method into 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine–treated (MPTP-treated) cynomolgus monkeys, a primate model for PD. We found that transplanted cells were able to function as DA neurons and could diminish Parkinsonian symptoms. This is the first report, to our knowledge, demonstrating the efficacy of transplantation therapy using ES cell–derived DA neurons in an experimental primate model of PD.

Results

Induction of neural progenitors from monkey ES cells. To enrich graftable neural progenitors, we first cultured monkey ES cells on PA6 stromal feeder cells, then detached the cells from the feeders for
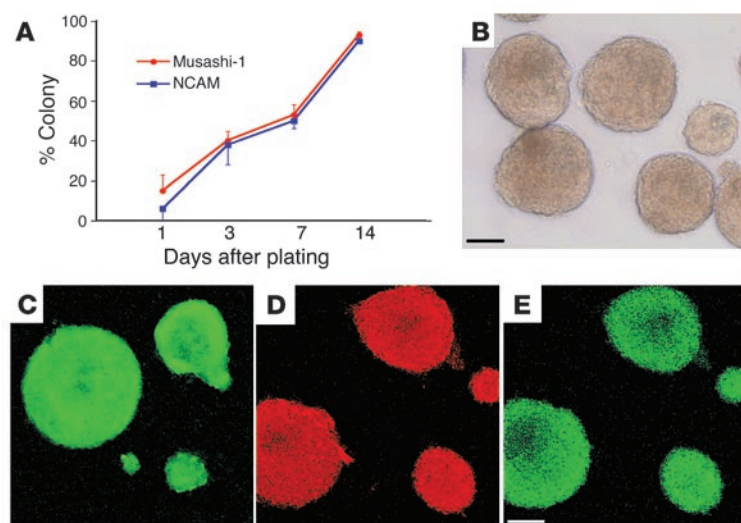
Figure 1
Neural progenitors induced from primate ES cells. (**A**) Time course of neural progenitor marker expression in monkey ES cells cultured on PA6 cells. (**B**) Detached ES cell colonies formed spheres similar to those of neural progenitor cells. (**C**–**E**) Spheres were immunoreactive for NCAM (**C**, green), Musashi-1 (**D**, red), and Nestin (**E**, green). Scale bar: 100 μm.

cells formed spheres morphologically resembling those formed by neural progenitor cells (Figure 1B). These spheres were positively stained with antibodies specific for neural progenitor cell markers NCAM, Musashi-1, and Nestin (Figure 1, C–E). To determine the potential of these cells to differentiate, we expanded the cells as spheres for 7 days, then induced differentiation by culturing the cells on poly-l-ornithine and laminin–coated (OL-coated) slides for 7 days. We removed the mitogens FGF2, EGF, and LIF from the medium, instead adding neurotrophic factors such as brain-derived neurotrophic factor and neurotrophin-3. Immunofluorescence analysis revealed that the cells differentiated into mature neural cells expressing the neuronal markers TuJ1 (52.8% ± 16.0% of DAPI) and Map2ab (38.3% ± 7.5% of DAPI), the astroglial marker glial fibrillary acidic protein (GFAP) (28.6% ± 17.6% of DAPI), and the oligodendroglial marker galactocerebroside C (GalC) (0.6% ± 0.4% of DAPI) (Figure 2, A–D and I). Further analyses demonstrated that these neurons derived from ES cells were immunoreactive for γ-amino butyric acid (GABA) (28.6% ± 10.7% of TuJ1), glutamate (14.3% ± 5.3% of TuJ1), choline acetyltransferase (ChAT) (0.7% ± 0.3% of TuJ1), and serotonin (3.3% ± 1.7% of TuJ1) (Figure 2, E–H and J). These results suggest that ES cell–derived spheres contain neural progenitor cells.

Effect of growth factors on differentiation of DA neurons. Effective treatment of PD requires substantial quantities of DA neurons. The percentage of tyrosine hydroxylase–positive (TH-positive) cells derived from neurospheres was only 5.4% ± 1.8% of the TuJ1-positive cells (Figure 2J). To increase the percentage of these cells, we examined the effects of various combinations of growth factors on neurosphere culture. The percentage of
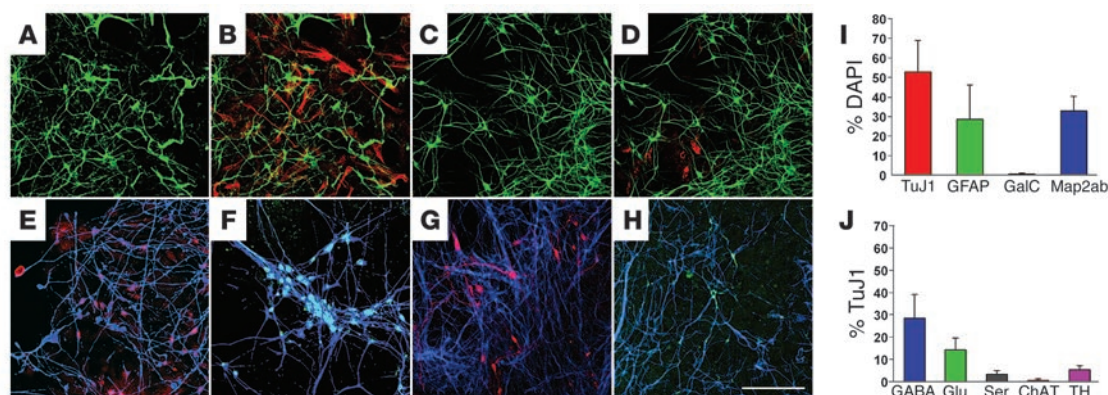
expansion as neurospheres. ES cells began to differentiate on PA6 cells, with cells immunoreactive for neural progenitor markers, such as N cell adhesion molecule (NCAM) and Musashi-1 (11, 14), emerging within three days. ES cells proliferated and differentiated on the feeder layer by forming colonies, and cells positive for neural markers increased in number until 2 weeks into the culture period. The percentages of the colonies including at least 1 NCAM- or Musashi-1–positive cell reached approximately 100% at 2 weeks of culture (NCAM = 90.4% ± 7.5%, Musashi-1 = 97.5% ± 4.6%, n = 100 from 3 independent cultures; Figure 1A). At this time point, 78.3% ± 7.5% of the total cells were immunoreactive for NCAM, 75.0% ± 15.4% for Musashi-1, and 72.3% ± 9.1% for another neural progenitor marker, Nestin (14). These results indicate that the majority of the ES cells were committed to the neural lineage by day 14 of culture on feeder layers.

In vitro characterization of neural progenitors derived from monkey ES cells. To further enrich neural progenitors, we detached the ES cells from the feeder layer on day 14, then continued to culture the cells on noncoated dishes in serum-free medium containing FGF2, EGF, and leukemia inhibitory factor (LIF). During the next few days, the

Figure 2
Expression of differentiated neural and neuronal subtype markers. Differentiated spheres were stained with antibodies against TuJ1 (**A** and **B**, green; **E**–**H**, blue), GFAP (**B**, red), Map2ab (**C** and **D**, green), GalC (**D**, red), GABA (**E**, red), glutamate (Glu; **F**, green), serotonin (Ser; **G**, red), and ChAT (**H**, green). Scale bar: 100 μm. The proportions of cells expressing differentiated neural (**I**) and neurotransmitter-related (**J**) markers are expressed as the mean ± SD of 3 independent cultures.
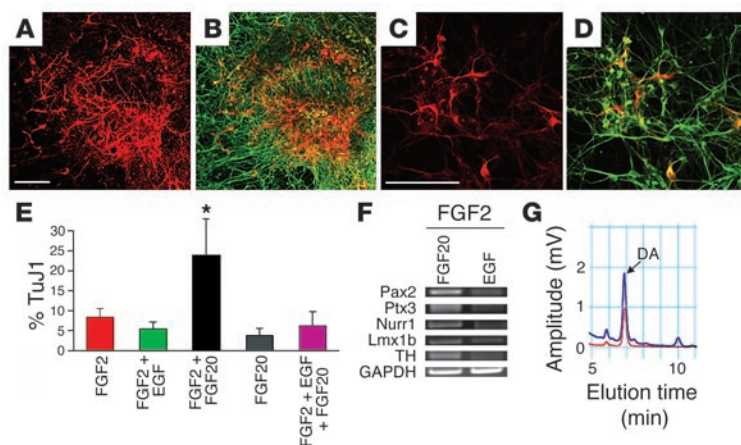
Figure 3
DA neurons differentiated from ES cell–derived neurospheres. (**A–D**) Differentiated spheres treated with FGF2 and FGF20 were stained with antibodies against TH (red) and TuJ1 (green). Scale bars: 50 μm. (**E**) The proportion of TH-positive cells to TuJ1-positive cells is expressed as the mean ± SD of 3 to 5 independent cultures. *$P < 0.05$. (**F**) RT-PCR for mesencephalic DA neuron markers *Pax2*, *Ptx3*, *Nurr1*, *Lmx1b*, and *TH* in cells treated with FGF2 and FGF20 (left) or FGF2 and EGF (right). (**G**) HPLC measuring concentration of dopamine released by SDIA- and FGF2- and FGF20-treated monkey ES cells in response to high K^+ depolarizing stimuli (blue line). Dopamine standard, red line.

TH-positive neurons out of total Tuj1-positive cells increased to 24% in the presence of FGF2 and FGF20 (Figure 3, A–E). Neither FGF2 nor FGF20 alone caused an increase in the number of TH-positive neurons. In the presence of FGF2, however, FGF20 increased the rate of TH differentiation in a dose-dependent manner (1 pg/ml = 3.8% ± 1.8% of TuJ1, 10 pg/ml = 8.8% ± 4.8% of TuJ1, 1 ng/ml = 24.3% ± 9.8% of TuJ1).

RT-PCR analyses showed that the cells derived from SDIA-induced neurospheres expressed mesencephalic DA neuron markers such as *Pax2*, *Ptx3*, *Nurr1*, *Lmx1b*, and *TH* (15, 16). The expression of these markers was more abundant in FGF2- and FGF20-treated cells than in FGF2- and EGF-treated cells (Figure 3F). Furthermore, FGF2- and FGF20-treated cells released 55.6 ± 19.9 pmol per 10^6 cells of dopamine in response to high K^+ depolarizing stimuli as assayed by HPLC ($n = 5$; Figure 3G). These results indicate that monkey ES spheres treated both with SDIA and with FGF2 and FGF20 generate a significant number of functional DA neurons in vitro.

Transplantation of DA neurons from ES-derived neural progenitors. To determine if the isolated TH-positive neurons function as DA neurons in vivo, ES cell–derived neurospheres were grafted into the putamen of the monkeys with MPTP-induced PD. In this primate model of PD, we administered MPTP intravenously to *Macaca fascicularis* (cynomolgus monkeys), hereafter *M. fascicularis*, and evaluated their behavior by scoring for neurological symptoms, such as motility and tremor (Table 1). Only the animals that exhibited stable deterioration for periods longer than 12 weeks were used for transplantation. ES cell–derived neurospheres used for grafting were prepared by treating with SDIA for 14 days and, subsequently, with FGF2 and FGF20 for 7 days, as described above. Using MRI images obtained for each monkey, we determined the necessary coordinates to stereotactically transplant the cells (300,000–600,000 cells per side) into the bilateral putamen. After injection, we continuously analyzed the behavior of postoperative monkeys by assessing neurological scores. We observed a slight recovery in the behavioral symptoms even in the sham-operated animals. At 10 weeks after transplantation, however, the mean scores of ES cell–transplanted monkeys significantly improved over the levels observed in sham-operated ones (Figure 4A, $n = 6$ and 4, respectively). In our evaluation of symptoms after transplantation, posture recovery was the most prominent improvement seen in ES cell–transplanted monkeys, with significant improvements in motility also observed. There were, however, no significant changes in head-checking movement. Consciousness was not disturbed in any preoperative animals, and no deterioration was observed postoperatively. In addition, none of the treated animals developed dyskinesia. Positron emission tomography (PET) at 14 weeks after transplantation revealed increases in ^{18}F-fluorodopa uptake at the putamen of the ES cell–transplanted animals (Figure 4, B and C).

After the PET study, animals were sacrificed and subjected to immunohistochemical analysis. Grafted cells, which were labeled by BrdU treatment during sphere culture, were detected in the putamen of ES cell–transplanted monkeys (7,996 ± 3,300 cells per side; Figure 5, A–C). DA neurons were detected by TH (Figure 5, B, C, E, and F; ref. 11) or dopamine transporter (DAT) (Figure 5, H and I; ref. 17) staining. An average of 2130 ± 645 TH-positive cells per side survived. Double labeling immunofluorescence microscopy revealed that 65.5% ± 4.3% and 50.3% ± 6.1% of the TH- and DAT-positive cells were immunoreactive for BrdU, respectively. Thus, the estimated number of TH/BrdU–double-positive cells was 1395 per side, 17.4% of the total of BrdU-positive cells. In contrast, in sham-operated monkeys, only a few TH-positive cells and scattered fibers were detectable in the putamen. No tumor formation or Ki-67–inducing reactivity was observed. As for other

Table 1
Neurological scores of MPTP-treated monkeys

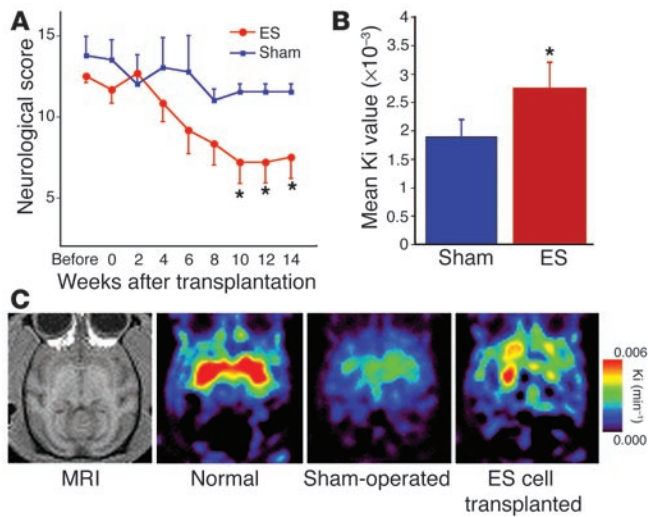| Behavior	Scores
Alertness	Normal, 0; reduced, 1; absent, 2
Head-checking movement	Present, 0; reduced, 1; absent, 2
Eyes	Normal, 0; reduced blinking, 1; eyes closed, 2
Posture	Normal, 0; mildly abnormal, 1; abnormal, 2; grossly abnormal, 3
Balance	Normal, 0; impaired, 1; frequent falling, 2; no movement, 3
Motility, at rest	Normal, 0; mild bradykinesia, 1; bradykinesia, 2; akinesia, 3
Motility, reaction to external stimuli	Normal, 0; mildly reduced, 1; reduced, 2; absent, 3
Walking	Normal, 0; mildly reduced, 1; reduced, 2; no walking, 3
Tremor	Absent, 0; mild/not always, 1; moderate, 2; severe, 3

Figure 4
Function of ES cell–derived neurospheres in MPTP-treated monkeys. Behavioral scores (**A**) and PET study (**B** and **C**) of ES cell–transplanted ($n = 6$) and sham-operated animals ($n = 4$). (**B**) Mean Ki values from entire putamen. (**C**) Increased ^{18}F-fluorodopa uptake in the putamen of ES cell–transplanted animals. All values are mean ± SD. *$P < 0.05$.

population of DA neurons from ES cell–derived neural progenitors. Using MPTP-treated monkeys as a primate model for PD, we analyzed the effect of administration of DA neurons generated from monkey ES cells in vivo. Behavioral studies and functional imaging revealed that the transplanted cells functioned as DA neurons, attenuating the MPTP-induced symptoms.

In our preparation of graftable cells, we made two major modifications to our previously published protocol (10, 22). First, we induced the formation of neurospheres, expecting an enrichment of neuronal progenitor cells. We detached the ES cells from the feeder layer on day 14 and cultured them on noncoated dishes. Under these conditions, the cells formed floating spheres composed of neural precursor cells. Since the serum-free culture medium was suitable for neural cell growth, any contaminating nonneural and PA6 cells were likely eliminated as a result of a low proliferation rate and/or adherence to the bottom of the dish. Our previous report (22) demonstrated that, when grafted into the brain, fully matured TH-positive neurons survived less efficiently than DA neuron progenitors induced by SDIA, probably due to their susceptibility to mechanical stress. This result is consistent with the fact that transplantation of mesencephalon tissues from early gestation stage embryos undergoing neurogenesis of DA neurons resulted in good survival of TH-positive cells (1300–18,000) and increased dopamine concentrations in the caudate nucleus of MPTP-treated monkeys, whereas these effects are not observed when mesencephalon tissues from later stages are transplanted (23). Thus, ES cell–derived neuronal progenitors competent to generate DA neurons appear to be more suitable for transplantation than DA neurons matured in vitro.

Another important modification to our previous protocol is the use of FGF2 and FGF20 treatment to enhance the generation of DA neurons. The percentage of TH-positive neurons generated from
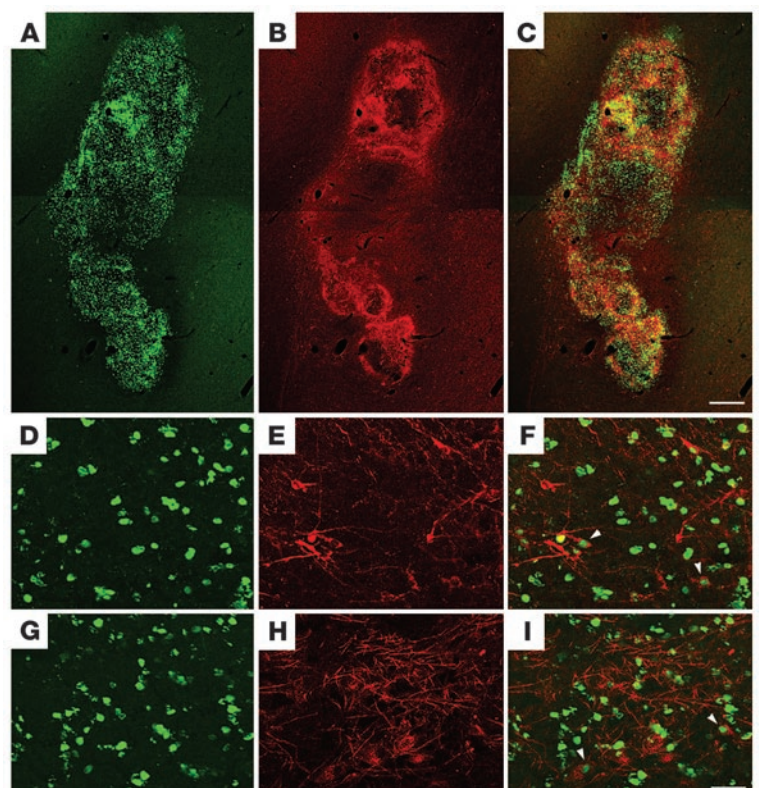
neuronal phenotypes, GABA-positive cells were detected in the graft at a slightly higher frequency than TH-positive cells, while few serotonin-positive cells were present (data not shown).

Discussion

Following initial work by Thomson et al. reporting a method for establishing primate ES cells (18), Suemori et al. (19) recently devised a similar scheme for generating *M. fascicularis* ES cells. Lee et al. recently reported a 5-step method to induce DA neurons from ES cells through the induction of neural progenitor cells from embryoid bodies (20). Transplantation of induced DA neurons derived from mouse ES cells improves the neurological symptoms of rats with a Parkinson-like syndrome induced by treatment with 6-hydroxydopamine (6-OHDA) (21). We have also reported a method of inducing DA neurons based on SDIA resulting from coculture of ES cells on a PA6 stromal feeder layer. By the use of this method, mouse ES cells are diverted to a neuronal fate with TH-positive DA neurons composing 30% of total TuJ1-positive neurons (10). Furthermore, this method produced similar results with *M. fascicularis* ES cells (11). In this study, we produced a highly enriched population of proliferating neural progenitors derived from SDIA-treated monkey ES cells. Furthermore, treatment of these cells with a combination of FGF2 and FGF20 induced the generation of a large

Figure 5
Survival of ES cell–derived DA neurons in the striatum. (**A**–**C**) Grafted cells (BrdU-labeled, green) survived and differentiated into DA neurons (TH-positive, red) along the needle tract (merged image **C**). Scale bar: 500 μm. (**D**–**I**) Colocalization (arrows in **F** and **I**) of BrdU (**D**, **F**, **G**, and **I**, green) and TH (**E** and **F**, red) or DAT (**H** and **I**, red) shows that graft-derived cells have dopaminergic character. Scale bar: 50 μm.

neurospheres was 5.4 ± 1.8% of TuJ1, much lower than that generated by culturing on PA6 cells (35%; ref. 10). This discrepancy most likely results from mechanical damage caused by detaching the cells or inappropriate culture conditions for spheres. To increase the number of DA neurons differentiated from neurospheres, we examined the effects of various additional growth factors. Ascorbic acid and Sonic hedgehog, which were used in the 5-step method (20), did not increase the proportion of TH-positive cells (data not shown). In contrast, FGF20 treatment in combination with FGF2 was able to efficiently increase the proportion of TH-positive cells. FGF20 is a secreted protein that is preferentially expressed in the substantia nigra pars compacta of the rat brain (13). The expression profile of FGF20 is quite different from that of other FGF family members, which suggests that FGF20 plays a unique role in the brain. Furthermore, recombinant FGF20 enhances the survival of primary DA neurons (13). FGF receptor–1c, the receptor through which FGF20 activates the mitogen-activated protein kinase pathway, is also preferentially expressed in the substantia nigra pars compacta (24). Our results raise the possibility that FGF20 in combination with FGF2 may support the survival or promote the proliferation of progenitors of DA neurons, resulting in the enrichment of DA progenitor cells in spheres. The mechanism by which this combined stimulation of FGF2 and FGF20 facilitates the production of DA neurons remains to be clarified.

FGF2 and EGF are reported to play different roles in the differentiation of neural precursors. Although FGF2 and EGF promote proliferation of neural precursor cells, the former promotes neuronal differentiation, while the latter induces glial differentiation (12, 25, 26). They also have different effects on the differentiation of embryoid bodies derived from human ES cells (27). In the present study using neural precursors derived from primate ES cells, FGF2 increased differentiation of ES cells into DA neurons, while EGF suppressed this process even in the presence of FGF2 and FGF20. It is possible that EGF interferes with the differentiation of SDIA-treated spheres into DA neurons directly or indirectly by promoting astroglial induction. Alternatively, EGF may stimulate proliferation or differentiation of a different cell population than that stimulated by FGF2. The differential effects of growth factors present an intriguing topic for future investigation.

In primate studies, functional neuroimaging is a useful tool for in vivo assessment of differentiation, survival, and functional integration of grafted cells. PET imaging of presynaptic targeting reagents such as fluoro-dopa, fluoro-metatyrosine, or 2β-carbomethoxy-3β-4-fluorophenyltropane (CFT) determines whether cells implanted in vivo have the molecular machinery necessary for dopamine synthesis and/or storage (28–30). In this study, we examined the uptake of fluoro-dopa at 14 weeks after transplantation. The significant increase in the mean Ki value in the putamen of ES cell–transplanted animals indicated that the grafted cells functioned as DA neurons. A postmortem examination of the ES cell–transplanted monkey in Figure 4C revealed that more TH-positive cells survived within the right putamen. This finding reflects the correlation of the PET results with the survival of DA neurons. For future studies, detailed analyses using additional tracers, including postsynaptic markers such as fluoro-raclopride, should allow for further understanding of the functional aspects of grafted cells. In this study, we detected significant differences in the mean Ki values from the entire putamen between the ES cell–transplanted monkeys and the sham-treated control monkeys 3 months after surgery. Widner et al. (8) reported that striatal

uptake of fluoro-dopa was unchanged 5 to 6 months postoperatively, but increased markedly at 12 to 13 and 22 to 24 months in patients who received fetal mesencephalic grafts. Freed et al. (4) reported an improved Ki value from the entire putamen 6 months after transplantation. Given the lengthier monitoring periods in these reports, our evaluation of mean Ki values at 3 months may still be premature; further PET studies at later time points may result in even greater changes.

MPTP is a neurotoxin that causes selective destruction of DA neurons in the substantia nigra pars compacta, inducing PD-like symptoms in primates (31, 32). Following repetitive intravenous injections of MPTP (approximately 17 mg in total per animal), monkeys stably exhibited PD-like symptoms more than 12 weeks before transplantation surgery. With a blind evaluation based on neurological scores, we detected significant behavioral improvements in the ES cell–transplanted monkeys 10 weeks after transplantation. Recently, 2 double-blind placebo-controlled clinical trials of fetal nigral transplantation (4, 7) demonstrated that younger patients and patients with mild symptoms improved after treatment, with behavioral recovery first observed in the 3 to 4 month period following surgery. In this study, posture and motility were the symptoms showing the most marked improvement. These results are comparable with clinical reports (4, 33) demonstrating improvements in rigidity and hypokinesia.

PET and immunofluorescence studies demonstrated that a substantial number of the grafted cells survived in the putamen to function as DA neurons. We transplanted 300,000–600,000 cells into each side of the brain in each monkey. The number of surviving cells detected by BrdU staining was approximately 8,000 per side. Thus, the survival rate of the grafted cells was 1.3% to 2.7%, although the actual value could be higher (discussed below). Through TH staining, the number of surviving DA neurons was shown to be approximately 4,300 per brain. In normal brains, there are no DA neuron cell bodies in the striatum, only fibers. Thus, these TH-positive cells were considered to be derived from the grafted ES cells. While the grafted cells were labeled by BrdU prior to transplantation, only 65% of these TH-positive cells were immunoreactive for BrdU. This may have resulted from incomplete labeling of the input cells; as the cells were treated with BrdU while being cultured as spheres, the labeling rate was not 100%, but 68.8%. In addition, grafted cells might proliferate in vivo, reducing the concentration of BrdU in the cells. Furthermore, intrinsic striatal TH-positive neurons may be recruited, as reported previously (34), which may explain the observation of a few TH-positive neurons even in control monkeys.

According to an earlier clinical report, the number of TH-positive cells in the postmortem brain of a PD patient was approximately 200,000 (35). In 2 recent double-blind trials, however, the number of surviving TH-positive cells was determined to be 50,000–240,000 (4, 7). Given that the volume of the monkey putamen is 10% of that of the human putamen (36, 37), it is likely that the required number of TH-positive cells in the monkey (*M. fascicularis*) brain is 5,000–24,000. The results of this study remain in keeping with observations made in human patients, suggesting that ES cells are a promising candidate for a donor source for cell transplantation treatment of PD. It should be noted, however, that the MPTP-treated monkey is a model of acute selective nigral destruction whereas human PD patients also experience progressive deterioration and pathological changes of other neural systems (15, 38, 39).

Although the results presented here encourage the development of strategies involving ES cell–derived neurons for treatment of neurological diseases, further studies will be needed to address the long-term efficacy and safety of using these cells. For instance, the low survival rate of the grafted cells or neurons is comparable to that noted in previous reports (40). To increase the number of viable DA neurons produced by grafts in vivo, we used DA neuron progenitors in the present study. Multiple-target grafting (41) is also a strategy that should be considered. Notably, we observed a number of GABA-positive cells in the graft, suggesting that other types of neurons and/or glial cells in the graft may contribute to both the differentiation and function of transplanted DA neurons (42). However, the optimal cellular composition of the graft remains to be determined. In addition, while previous studies with rodents have demonstrated that tumor formation can be associated with ES cell grafts (43–45), we did not observe tumor formation or Ki67-positive cells within the first 3 months after transplantation. In the future, however, it will be important to examine late tumor formation as well as the possible long-term effects of ES cell grafts on motor behavior. It will also be necessary to use non–TH-positive cells in control grafts to exclude the possibility that the effects of this treatment are mediated by non-DA cells.

Finally, we would like to emphasize that our system (MRI, surgery, PET, etc.) is applicable to humans. Previous work has shown that monkey ES cells have characteristics similar to those of human ES cells (18, 19, 46). In addition, it was recently demonstrated that neural precursors induced from human ES cells were able to survive in rodent brains (47, 48). The SDIA method is applicable to human ES cells, allowing for enrichment of DA progenitors (unpublished data). These results suggest that transplantation using ES cells as a clinical therapy for PD is approaching the point of technical feasibility. Two recent double-blind, sham surgery–controlled trials of embryonic mesencephalic transplants for the treatment of PD, however, showed only modest improvement (4, 7), suggesting the potential limits of cell transplantation. Many basic issues, especially regarding stem cell therapy, remain to be resolved (38). Before the clinical application of human ES cell transplantation can be attempted, extensive studies assessing the safety and efficacy of ES cell transplantation in monkey models will be necessary.

Methods

Maintenance of primate ES cells. Cynomolgus monkey ES cell lines were established and their pluripotency confirmed by teratoma formation in mice with severe combined immunodeficiency, as previously described (19). Undifferentiated ES cells were maintained on a feeder layer (i.e., STO) of embryonic fibroblasts treated with mitomycin C (Wako Pure Chemical Industries Ltd.) in DMEM (Sigma-Aldrich)/F-12 (Invitrogen Corp.) supplemented with 0.1 mM 2-mercaptoethanol (2-ME) (Sigma-Aldrich), 1,000 units/ml LIF (Chemicon International), 20% knockout serum replacement (KSR; Invitrogen Corp.), and 4 ng/ml FGF2 (Upstate Biotechnology Inc.). ES cells were subcultured using 0.25% trypsin (Invitrogen Corp.) in PBS with 20% KSR and 1 mM $CaCl_2$ (Wako Pure Chemical Industries Ltd.) as described (11, 19).

Induction of neural progenitors from primate ES cells. PA6 cells plated on type I collagen–coated chamber slides (BD) or gelatin-coated dishes (gelatin from Sigma-Aldrich; dishes from BD) were used as a feeder cell layer. To avoid contamination by incidentally differentiating cells, we manually selected undifferentiated ES cell colonies with stem cell–like morphology (tightly packed cells exhibiting a high nucleus to cytoplasm ratio). Undifferenti-

ated ES cell colonies were washed twice with Glasgow minimum essential medium (GMEM) (Sigma-Aldrich) supplemented with 10% KSR, 1 mM pyruvate (Sigma-Aldrich), 0.1 µM nonessential amino acids, and 0.1 mM 2-ME. After trypsinization, partially dissociated ES cell clumps (10–50 cells per clump) were plated on PA6 cells at 1000 clumps per 10-cm dish. Cells were then cultured in GMEM supplemented with 5% KSR, 2 mM glutamine, 0.1 mM nonessential amino acids, 1 mM pyruvate, and 0.1 mM 2-ME for 2 weeks. Differentiated colonies were detached from feeder cells using a papain dissociation system (Worthington Biochemical Corp.). Isolated colonies were cultured in neurobasal medium (Invitrogen Corp.) supplemented with B27 supplement (Invitrogen Corp.), 20 ng/ml FGF2, and 20 ng/ml EGF (R&D Systems) for 1 week. In the experiments examining the effecs of growth factors on neurosphere culture, the indicated concentrations of FGF20 (1pg/ml, 10 pg/ml and 1 ng/ml) were added to medium with or without FGF2 and EGF. In order to evaluate the expression of neural progenitor cell markers by each cell, spheres were incubated with papain at 37°C for 10 minutes and then mechanically dissociated into single cells. After incubation with a papain inhibitor, the dissociated cells were plated on OL-coated slides (OL from Sigma-Aldrich) at a density of 10^5 cells/cm². After 16 hours, they were fixed in 4% paraformaldehyde (Sigma-Aldrich) and evaluated by immunofluorescence.

Differentiation of neural progenitor cells. Spheres were isolated manually and plated on OL-coated slides in neurobasal medium to which had been added B27 supplement, 20 ng/ml brain-derived neurotrophic factor (Sigma-Aldrich), and 20 ng/ml neurotrophin-3 (Sigma-Aldrich). After 1 week of culture, spheres were fixed or used for additional experiments.

RT-PCR analysis. Total RNA was isolated from differentiated cells using a TRIzol kit (Invitrogen Corp.) according to the manufacturer's instructions. cDNA synthesis was carried out using the SuperScript Double Stranded cDNA Synthesis kit (Invitrogen Corp.). PCR was performed using KOD-plus polymerase (Toyobo Co.), with the following cycling conditions: 30 seconds at 94°C, 30 seconds at 60.5°C, 60 seconds at 68°C × 30 cycles for Pax2, Ptx3, Nurr1, and GAPDH; and 30 seconds at 94°C, 30 seconds at 66°C, 60 seconds at 68°C × 30 cycles for Lmx1b and TH in a thermal cycler (Astec Co.). The experiments were repeated 4 to 7 times for confirmation, and PCR products were sequenced to rule out false positives. The primers used are as follows: *Pax2*, TGTGTCAGCAAAATCCTGGGCAGGT and TGCTGAACTTTGGTCCGGATGAT; *Ptx3*, TTCGCCTTCAACTC-GGTCAACGT and CCCAGGGTCTGAAAGGGGTG; *Nurr1*, CTCCCAGAGGGAACTGCACTTCG and CTCTGGAGTTAAGAAATCG-GAGCTG; *Lmx1b*, GCAGCGGCTGCATGGAGAAGATCGC and GGTTCT-GAAACCAGACCTGGACCAC; *TH*, GACTGCTGCCACGAGCTGCTGGG and TCTTGGTAGGGCTGCACGG; and *GAPDH*, GTGAAGGTCGGAGT-CAACG and GGTGAAGACGCCAGTGGACTC.

Dopamine release assay. Spheres treated with FGF2 and FGF20 were plated onto 35mm OL-coated dishes at a density of 50 spheres per dish and made to differentiate for 1 week. Then, after being rinsed in a low-KCl (4.7 mM) solution, the cells were incubated in 1 ml of a high-KCl solution (20 mM HEPES-NaOH, pH 7.4, 85 mM NaCl, 60 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 11 mM glucose) for 15 minutes. Concentrations of dopamine were determined by HPLC using a reverse-phase column and an electrochemical detector system (HTEC 500; Eicom Corp.) as previously described (49).

Animal model. Adult male cynomolgus monkeys (*M. fascicularis*) weighing 2.5–3.5 kg were given intravenous injections of MPTP HCl (0.4 mg/kg as free base, Sigma-Aldrich) twice a week until persistent Parkinsonian behavioral disturbances, such as tremor, bradykinesia, and impaired balance, became evident. Animals were given an average of 14.7 MPTP shots and exhibited stable Parkinsonism for approximately 30 days. To prevent any possibility of spontaneous recovery, only those monkeys that presented

stable deterioration for a period greater than 12 weeks were used for transplantation experiments. All animals were fed with commercial pellets and fresh fruits and had free access to clean water. Monkeys were cared for and handled according to Guidelines for Animal Experiments of Kyoto University and the National Cardiovascular Center (Osaka, Japan) and the Guide for the Care and Use of Laboratory Animals from the Institute of Laboratory Animal Resources (ILAR; Washington, DC, USA).

MRI. For accurate orientation of the putamen, animals ($n = 10$; 6 for ES transplanted and 4 for sham-operated group) were subjected to MRI examination using a 3.0 Tesla Signa system (General Electric). Following anesthesia by intramuscular injection with ketamine hydrochloride (15 mg/kg; Sankyo Co.) and xylazine (1.5 mg/kg; Boehringer Ingelheim Vetmedica Inc.), animals were positioned into the magnet using an MR-compatible headholder. T1-weighted images were used for further examination.

Transplantation. Following anesthesia with pentobarbital (7.5 mg/kg, intramuscularly [i.m.]; Dainippon Pharmaceutical Co.) and ketamine (10 mg/kg, i.m.), monkeys were fixed in a surgical frame (Narishige SN-1N; Narishige Co.). Neural progenitors from monkey ES cell cultures in two 6-cm dishes (300,000–600,000 cells per dish) were collected for each animal. While the ES cells were expanding as spheres, BrdU (5 μg/ml; Sigma-Aldrich) was added to the culture medium for 7 days to label the ES cells. Using an electric injector (Muromachi Kikai Co.), we transplanted donor cells into the putamen bilaterally, using the MRI findings for each monkey and the *M. fascicularis* brain atlas (36, 50). To cover the mid to posterior putamen, 3 targets that were 2 mm apart in anterior-posterior position and 1–2 mm in medial-lateral position were set, and 4 injections (1 μl/60 seconds for each injection) were made along each tract. After surgery, all animals were given antibiotics for 1 week and a daily immunosuppressant (cyclosporin A, 10 mg/kg, i.m.; Carbiochem) until sacrifice. Monthly blood analyses performed over the course of the experiment confirmed the levels of circulating cyclosporin A, which averaged 393 ng/ml. In control monkeys undergoing sham operations, the equivalent volume of culture medium was administered without cells. Afterward, antibiotics and immunosuppressants were injected as for the cell-grafted monkeys.

Immunofluoresence study. For in vitro studies, cells were fixed in 4% paraformaldehyde for 10 minutes for microscopic observation. For in vivo studies, after administration of deep anesthesia with pentobarbital and ketamine, monkeys ($n = 10$; 6 for ES cell–transplanted and 4 for sham-operated group) were transcardially perfused with 4% paraformaldehyde. Excised brains were frozen and cut with a microtome at a 50-μm thickness. Sections were then stained by the free-floating method. Slides were first incubated in 0.3% Triton X with 5% skim milk in PBS for 30 minutes. Samples were then incubated with one of the following primary antibodies: rabbit anti-NCAM (1:300; Chemicon Inc.), rat anti–Musashi-1 (1:200; gift from H. Okano, Keio University, Japan), mouse anti-Nestin (1:300; Chemicon Inc.), mouse anti-TuJ1 (1:300; BabCO), rabbit anti-GFAP (1:1000; Chemicon Inc.), mouse anti-Map2ab (1:250; Sigma-Aldrich), rabbit anti-GalC (1:100; Chemicon Inc.), rabbit anti-glutamate (1:200; Chemicon Inc.), goat anti-ChAT (1:1000; Chemicon Inc.), mouse anti-GABA (1:1000; Sigma-Aldrich), mouse anti-BrdU (1:200; BD), rabbit anti-TH (1:60; Chemicon Inc.), rabbit anti-serotonin (1:2000; DiaSorin Inc.), or rat anti-DAT (1:100; Chemicon Inc.) in 2% skim milk in PBS overnight at 4°C. After 3 washes in PBS, samples were incubated with the appropriate secondary antibodies for 1 hour at room temperature: FITC-labeled anti-mouse Ig (1:200; Jackson Immunoresearch Laboratories Inc.) and Cy3-labeled anti-rabbit Ig (1:200; Jackson Immunoresearch Laboratories Inc.). For nuclear staining, 200 ng/ml of 4′,6′-diamidino-2-phenylindole (DAPI) was added in the final wash. After being washed in PBS, samples were mounted and analyzed using a Fluoview FV300 laser confocal microscope (Olympus Optical Co.). For in vitro studies, cells in

5 randomly selected fields, each of which included 100–500 cells, were counted for 3 to 5 independent cultures. For in vivo studies, the number of immunoreactive cells was quantified in every sixth section throughout the graft and surrounding tissue. Statistical analysis was performed by one-way ANOVA and post hoc multiple comparison by the Dunn test. A P value of less than 0.05 was considered significant.

Behavioral assessment. Parkinsonian behavior was evaluated using a rating scale previously proposed by Akai et al. (51), with modifications (Table 1). To prevent subjective biases, the evaluation was performed by a trained examiner who was not involved in the culturing and transplantation of the cells and not informed of the specific procedure to which each monkey was subjected. Student's t test was used to compare the 2 groups, and a P value of less than 0.05 was considered significant.

PET. To evaluate the in vivo DA function of the grafted cells, PET scans using ^{18}F-fluorodopa were performed on each animal prior to perfusion. Under generalized anesthesia with a continuous infusion of propofol (4 mg/kg/h; Zeneca Pharmaceuticals) and vecuronium-bromide (0.25 mg/kg/h; Boehringer Ingelheim Vetmedica Inc.), analyses were performed using an ECAT EXACT HR+ PET scanner (Siemens/CTI). After intravenous injection of 185MBq of ^{18}F-fluorodopa, brain radioactivity was assessed for 90 minutes in animals that had received carbidopa (10 mg/kg) 30 minutes prior to the PET scan. Parametric images of the dopamine-irreversible metabolic rate of Ki (min^{-1}), considered to be a measure of presynaptic DA function, were generated using the time course of radioactivity in each voxel by multiple-time graphical analysis (52) using the bilateral occipital lobes as a reference region. The ^{18}F-fluorodopa Ki image was coregistered to the corresponding T1-weighted MRI image, which was obtained by an inversion-recovery prepared fast spoiled gradient recalled acquisition in the steady state (IR-FSPGR) sequence (Tr = 9.4, TI = 600, TE = 2.1 in msec) using a 3.0 Tesla Signa System (General Electric) and realigned to a standard space of *M. fascicularis* (36, 50). DA function was evaluated by visual inspection of Ki images and by quantitative Ki values in the bilateral striatum identified by the corresponding MRI image. This evaluation was performed in a blind manner to ensure objectivity. Student's t test was used to compare the results.

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