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). Undifferentiated 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/cm^2. 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*, TTCGCCTTCAACTCGGTCAACGT and CCCAGGGTCTGAAAGGGGTG; *Nurr1*, CTCCCAGAGGGAACTGCACTTCG and CTCTGGAGTTAAGAAATCGGAGCTG; *Lmx1b*, GCAGCGGCTGCATGGAGAAGATCGC and GGTTCTGAAACCAGACCTGGACCAC; *TH*, GACTGCTGCCACGAGCTGCTGGG and TCTTGGTAGGGCTGCACGG; and *GAPDH*, GTGAAGGTCGGAGTCAACG 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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