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厚生労働科学研究費補助金

医療技術実用化総合研究事業

ヒト生体由来多能性幹細胞（Muse細胞）の再生医療への応用に向けた安全性・有効性の検証

平成２４年度　総括研究報告書

研究代表者　出沢　真理

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研究代表者　出沢真理　東北大学大学院医学系研究科・教授

研究要旨　Muse 細胞は成人ヒトの骨髄、皮膚などの間葉系組織から多能性幹細胞マーカーと間葉系マーカーの二重陽性細胞として採取可能な生体由来の多能性幹細胞であり、腫瘍性を持たない。3 胚葉性のあらゆる細胞に分化する能力を持ち、そのまま生体内に投与すると損傷部位に生着し、組織に応じて機能的細胞に分化して様々な臓器の再生をもたらす。Muse 細胞を迅速に臨床試験を実現するために、誘導しないそのままの Muse 細胞懸濁液を疾患部位に投与する「医薬品」として開発することを本研究の目的とし、ターゲット臓器を肝臓に絞り非臨床有効性試験、細胞調製の最適化、Muse 細胞製剤の規格の設定、安全性検証等を行った。本年度は急性肝障害モデルを中心に研究を行ったところ、Muse 細胞は肝組織への生着、分化による組織修復を担うが、Muse 以外の非 Muse 細胞にはこのような能力がないことが分かった。Muse 細胞の核型検査を行ったところ、変異等異常は無いことが分かった。Muse 細胞の実用化を推進することにより、幅広い疾患を対象に安定的な効果をもたらす細胞治療が世界に先駆けて実現されると期待される。

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A．研究目的

間葉系幹細胞は 3 胚葉性にまたがる多様な細胞に分化するため、多能性幹細胞が内包されると推察されていた。また一部の細胞は生体内で損傷部位に生着し、組織修復を行うことも報告されていた。我々は間葉系幹細胞の多様な分化や組織修復を説明する多能性幹細胞 Muse 細胞を発見した。Muse 細胞は多能性幹細胞としての特性を備えているが腫瘍性が無いところが最大の利点である。

Muse細胞は成人ヒトの骨髄、皮膚、脂肪などの間葉系組織からヒトES細胞のマーカーであるSSEA-3と間葉系マーカーであるCD105二重陽性細胞として同定できる。Muse細胞は1細胞から自発的に3胚葉性の細胞に分化する能力を有する。採取後、誘導せずにそのまま生体内に投与すると損傷部位に生着し、機能的細胞に分化して様々な組織再生をもたらす。特定の誘導をかけることによって神経、肝細胞、骨細胞などに90%以上の高い効率で誘導可能である。

Muse細胞はいくつかの利用方法が想定できるが、迅速に臨床試験を実現するためには、誘導しないそのままのMuse細胞懸濁液を疾患部位に投与する「医薬品」として開発が最も現実的であると考えられる。Muse細胞はすでにヒトに移植されている骨髄移植や間葉系細胞移植に含まれているとはいえ、精製した細胞を投与する際の安全性・有効性評価は必須である。本研究ではPMDAの審査官として薬事承認審査等の経験を有する浅田隆太と民間企業（株）Clio社が研究分担者として参画し、実用化を目指した検証を行う。

検証のターゲット臓器として肝臓を選択し、①有効性検証の指標設定、②非臨床有効性試験（急性と慢性の肝疾患モデルを用いた有効性検証、用量探索、投与速度の最適化）、③細胞調

製の最適化、④Muse細胞製剤の規格の設定、⑤非臨床安全性評価を実施する。さらに薬事戦略相談を適宜利用して、安全性が高く再生効果を有するMuse細胞を出来る限り早く臨床試験に移行できるように進める。

B．研究方法

【投与速度の最適化】

ヌードマウスを用いてヒト Muse 細胞の母集団である線維芽細胞を用いておこなった。20,000細胞を 30 秒、5 分、30 分の速度で電動マイクロシリンジを用いて投与し、呼吸不全などの有害事象を観察し、3 日間に肺塞栓の有無を組織学的に評価した。

【急性・慢性肝障害モデルの作成】

ヒト細胞を拒絶しない免疫不全動物のSCIDマウスを使用する。急性肝障害モデルは腹腔内に四塩化炭素CCl4を0.5, 1.0 1.5 mg/Kgの量で投与し、モデルとして最適の作成量を決定する。慢性モデルは週に２回、1.0 mg/Kg の分量を4週間にわたって投与し作成する。ヒトMuse細胞は骨髄間葉系幹細胞からFACSでSSEA-3/CD105ダブル陽性細胞として採取する。FACSによってSSEA-3陰性/CD105陽性細胞を非Muse細胞として採取する。

【ヒトMuse細胞の有効性評価、用量探索試験】

尾静脈投与するヒトMuse細胞あるいは非Museの細胞数は5000、20,000、40,000細胞の3点を設定し、急性・慢性モデルそれぞれに尾静脈から投与する。本年度は20,000細胞を中心に実験を行った。

移植前、及び移植後、day1, 5, 9, 16, 23, 30において血清アルブミン量、総蛋白量、ビリルビン値の他、体重、一般血液、血液生化学、全身状態、組織学的検討による線維化領域の算出、などによって有効性評価行う。評価時期は急性モデルは単回の四塩化炭素投与から30日後、慢性モデルは全ての四塩化炭素の投与を終えてから4週後に評価を行う。

さらに組織学的検討を行う。組織用にはlentivirus GFPをあらかじめ標識したMuseあるいは非Museを投与し、抗GFP抗体、抗ヒトミトコンドリア抗体によってヒトMuse,非Museの検出を可能とした。さらに肝臓の分化マーカーであるHeppar 1、human albumin, human anti-trypsinに対する抗体を用いてSCIDマウス肝臓内で生着したヒト細胞の分化を確認した。

【非臨床安全性評価】

GLP を準拠しガイドラインに従い、以下の非臨床試験を行う。Lonza 社より購入した健康成人に由来する骨髄由来の間葉系幹細胞（MSC）と、臨床使用と同じ方法で MSC より分離した Muse 細胞の核型検査を実施し、製造工程が Muse 細胞の核型に与える影響を検討する。本試験は、WHO Expert Committee on Biological Standardization (WHO: World Health Organization) の方法および ATCC (American Type Culture Collection) の培養細胞品質検査方法を参考に、「厚生省令第 21 号「医薬品の安全性に関する非臨床試験の実施の基準に関する省令」（平成 9 年 3 月 26 日、一部改正 厚生労働省令第 114 号 平成 20 年 6 月 13 日）を遵守して一般財団法人食品薬品安全センター 秦野研究所で実施された。

Muse 細胞の分離については、これまでの非臨床試験では、自動細胞解析分離装置である Fluorescence Activated Cell Sorter（FACS）を用いてきたが、FACS はレーザービームで細胞を傷害する可能性があること、また、臨床応用されていないことを勘案し、海外において臨床応用されている、自動磁気細胞分離装置である CliniMACS®を臨床で用いることとする。今後、実施する非臨床試験に用いる Muse は自動磁気細胞分離装置である autoMACS®を用いて、分離することとする。MACSは分離能がFACS に比べ、劣るが、細胞傷害性は低いと考えられる。また、MACS を用いて、3 回カラムを通すことにより、陽性率が 7～8 割の Muse 細胞を得ることができ、得られる細胞数も多い。

（倫理面への配慮）

東北大学と山口大学の遺伝子組換え実験安全専門委員会と動物実験専門委員会の指針に従って

研究計画書を提出し、機関承認を得た後に実験を実施しており、承認を得た計画のみを実行する。

ヒト間葉系細胞はスイス Lonza 社やアメリカの ATCC などから購入した細胞を用いる。ヒト各種組織、臓器などは同じく購入によって入手したものを実験に用いる。

実験におけるヒト細胞使用に関しては「東北大学医学部・医学系研究科倫理委員会」より「ヒト骨髄および臍帯組織由来間葉系細胞の解析研究（2008－82 号）」で承認をすでに受けている。

遺伝子導入実験は「東北大学　遺伝子組み換え実験計画承認」を得ている（研研76－20－35号）。また動物実験委員会の承認に関しては2011医動－282、2011医動－283、2011医動－284、2011医動－285にて承認を得た上で実験を行っている。

C．研究結果

【投与速度の最適化】

9 匹のヌードマウスすべてにおいて，morbidity と mortality はみられなかった。組織学的評価でも，すべてのマウスで肺塞栓はみられなかった．3 種類の速度を設定し、投与速度を行ったが，経尾静脈細胞移植はすべての条件において安全であった．従って本実験では 30 秒での細胞投与の速度を採用することとした。

【急性肝障害モデルの作成と有効性評価、用量探索試験】四塩化炭素の量を0.5，1.0 1.5 mg/Kgで投与したところ、いずれの条件でも体重，血中GOT、GPT、総ビリルビン，総蛋白，アルブミンは一過性(一日後~10日前後まで）に増悪するものの，すべてのパラメータで30日前後には正常に戻ることが確認された。従って一回だけの四塩化炭素投与による急性障害はマウスにおいては一月程度で自然に再生する。組織学的な検討においても同様の結果が確認された。そこで四塩化炭素 1.5ml/kg腹腔内投与によるマウス肝障害モデルで移植実験を行い、評価項目は移植細胞のアルブミン分泌能，障害肝への生着・分化効率を中心にすることとした。

ヒト骨髄由来Muse細胞、非Muse細胞をそれぞれFACSで分離した2万細胞を四塩化炭素投与2日目にSCIDマウス尻静脈より投与した。その結果、Muse細胞を投与した群ではtotal ビリルビンの上昇がマイルドに抑えられた他は、体重，血中GOT、GPT、総ビリルビン，総蛋白，アルブミンは一過性に増悪し、14日前後から正常近くに戻ることにおいて有意差は見られなかった。

ただ顕著な結果として、lentivirus-GFPで標識したヒトMuse，非Muse細胞の生着を移植30日後で評価したところ、非Muse細胞はほとんど肝臓組織内に残っておらず、従って肝細胞への分化も見られなかったが、Muse細胞は中心静脈などの血管系を中心に細胞が組織内に入り、生着し、しかもHeppar 1，human albumin，human anti-trypsinなど、肝臓の機能的なマーカー陽性を示していた。移植した肝臓でhuman albuminのシグナルがRT-PCRで検出され、さらにマウスの末梢血を採取しWestern blotで解析するとhuman albuminが検出された。

さらにMuseと非Museの生着の有無を確認するために移植肝臓のゲノムを採取し、human prostaglandin E receptor2とマウスの同受容体を検出することによって、ヒトゲノムの有無を調べた。移植30日においてMuseを移植した肝臓からはヒトゲノムが検出されたが非Museでは検出されなかった。

このことからMuse細胞は血管に投与されただけで障害肝臓組織に入り、肝細胞に分化する能力を持つが非Muse細胞にはそのような機能は持ち合わせていないことが示唆された。

肝臓はもともと再生力のある臓器であり、特に急性肝障害モデルでは移植初期にしか血液データーの差が見られないが、組織学的には修復能力に差があり、Muse細胞は肝修復に寄与することが分かった。

【慢性肝障害モデルの作成と有効性評価、用量探索試験】

四塩化炭素を週に2回、一回の分量を1.0 mg/Kg 、4週間にわたって投与することによっ

て、肝臓の顕著な線維化、委縮が起こることを確認している。現在Muse細胞、非Muse細胞を20,000細胞移植し、経過を観察中である。

【非臨床安全性評価】

hMSC-L5 のギムザ染色による 100 核板の染色体数分析の結果、最頻値は正常ヒト細胞と同じ 46 本（頻度 92%）であり、それ以外の核板は偽二倍体構成が 7 核板（45 本が 4 核板、47 本が 3 核板）と、染色体数 92 本の四倍体構成が 1 核板であった。また、観察した 100 核板のうちに構造異常が観察されたものは無かった。染色体分染による 10 核板の核型分析の結果、10 核板ともにバンドパターンが正常なヒト女性染色体と一致し、欠失、転座、重複など、染色体レベルで確認できる異常は存在しなかった 。染色体分染、ギムザ染色による 100 核板の観察においても、染色体数の変化は無いと判断された。

D．考察

間葉系幹細胞は肝硬変、心筋梗塞、脊髄損傷、脳梗塞、変形性関節症などの多岐にわたる疾患において、ドイツ、イギリス、アメリカを含め、世界中で臨床試験が展開され一定の効果を挙げている。もたらす効果として、間葉系幹細胞の産生するサイトカインによる保護効果の他に、投与したごく一部の細胞が損傷を受けた組織に生着して分化し、失われた細胞をreplaceする再生効果が報告されている。ただサイトカイン効果は一過性であり、持続的な機能回復は細胞補充の再生効果が担う。このような細胞の探索が世界中で進められていたが、今回間葉系幹細胞の数パーセントを占める多能性を持つMuse細胞が再生効果を担い、それ以外の非Muse細胞は組織を修復し再生させる効果が無いことが示唆された。

さらに核型検査によってMuse細胞は正常なヒト染色体と一致し、欠失、転座、重複など、染色体レベルで確認できる異常は存在しなかったことはMuse細胞の安全性の面から大きな結果である。

次年度は慢性モデルを積極的に推進するとともに、用量探索試験、細胞調製の最適化，Muse細胞製剤の規格の設定、安全性薬理試験、造所要試験を実施する。

E．結論

速度投与の最適化検証においては数万の細胞を 30 秒で投与しても肺塞栓を含めた有害事象は生じないことが明らかとなった。

また SCID マウス急性肝障害モデルへのヒト Muse、非 Muse 細胞移植を行ったところ、Muse 細胞は 30 日後において肝組織に残り、生着し、肝細胞分化マーカーを発現し、albumin 等機能的な分子を産生する能力を有することが分かった。一方非 Muse 細胞の生着はほとんど認められなかったことから、間葉系幹細胞の組織修復・再生作用は Muse 細胞によるものであり Muse 以外の細胞は寄与しないことが分かった。

Muse 細胞の核型検査を行ったところ、変異等異常は無いことが分かった。

Muse 細胞の実用化を推進することにより、幅広い疾患を対象に安定的な効果をもたらす細胞治療が世界に先駆けて実現されると期待される。

F．危険情報

無し

G．研究発表

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H. 知的財産権の出願・登録状況

1．特許取得
無し

2．実用新案登録
無し

3．その他
無し

別紙４

研究成果の刊行に関する一覧表

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Autologous mesenchymal stem cell–derived dopaminergic neurons function in parkinsonian macaques

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A cell-based therapy for the replacement of dopaminergic neurons has been a long-term goal in Parkinson's disease research. Here, we show that autologous engraftment of A9 dopaminergic neuron-like cells induced from mesenchymal stem cells (MSCs) leads to long-term survival of the cells and restoration of motor function in hemiparkinsonian macaques. Differentiated MSCs expressed markers of A9 dopaminergic neurons and released dopamine after depolarization in vitro. The differentiated autologous cells were engrafted in the affected portion of the striatum. Animals that received transplants showed modest and gradual improvements in motor behaviors. Positron emission tomography (PET) using [^{11}C]-CFT, a ligand for the dopamine transporter (DAT), revealed a dramatic increase in DAT expression, with a subsequent exponential decline over a period of 7 months. Kinetic analysis of the PET findings revealed that DAT expression remained above baseline levels for over 7 months. Immunohistochemical evaluations at 9 months consistently demonstrated the existence of cells positive for DAT and other A9 dopaminergic neuron markers in the engrafted striatum. These data suggest that transplantation of differentiated autologous MSCs may represent a safe and effective cell therapy for Parkinson's disease.

Introduction

Cell-based therapies are expected to replace the missing dopaminergic neurons and to restore the motor function in patients with Parkinson's disease (PD) (1). Early studies on cell-based therapies used fetal midbrain tissue containing dopaminergic neurons as a cell source and suggested potential therapeutic effects in PD (for review, see refs. 2, 3). However, limited availability and ethical considerations relating to the use of fetuses pose limitations for practical use. Bone marrow–derived mesenchymal stem cells (MSCs), a type of adult stem cells, have trophic effects (4) and a differentiation spectrum that crosses oligolineage boundaries (5), offering the potential for use in autologous cell therapy, with low risk of tumorigenesis (6). The MSCs have been already tested for cell therapy in PD model rodents (7–9) and even in patients with PD (10). However, they have shown poor performance for restoration of motor function, potentially due to limited spontaneous differentiation (11) or facilitated apoptosis (12, 13) of MSCs. Recent studies of fetal midbrain graft have suggested that better outcomes could be obtained if the graft consisted of well-differentiated A9 dopaminergic neurons (14–16), the most severely damaged neuronal type in PD (17). Therefore, differentiation of MSCs into desired cells, such as A9 dopaminergic neurons, would probably provide effective functional restoration in PD.

Recently, it was shown that MSCs could be artificially directed to differentiate into several specialized cell types, including those in nervous tissues (18–21). Previously, we reported that dopamine-producing cells could be induced from MSCs (MSC-DP cells) by introduction of a Notch1 intracellular domain–containing (NICD-containing) plasmid, followed by cytokine stimulation with bFGF, forskolin, ciliary neurotrophic factor (CNTF), and glial cell line–derived neurotrophic factor (GDNF) (20, 21). The differentiated cells were positive for markers of dopaminergic neurons, such as tyrosine hydroxylase (TH) and the dopamine transporter (DAT), and had an ability to release dopamine after depolarization by potassium stimulation. When rat and human MSC-DP cells were transplanted into the striata of PD model rats, integration of TH$^+$ and DAT$^+$ cells and functional recovery in motor behaviors were confirmed (20). Subsequent development of a spermine-pullulan–mediated reverse transfection method allowed us to induce MSC-DP cells more safely and efficiently than before from MSCs of macaque monkeys (21), an animal species frequently used for preclinical trials of PD (22–27).

To test the scalability of MSC-DP cell–based therapy in primates in this study, monkey MSC-DP cells were characterized in detail using specific markers and evaluated for their longitudinal effects after they were engrafted into hemiparkinsonian monkeys using behavioral tests and positron emission tomography (PET). The MSC-DP cells, prepared autologously from the bone marrow of

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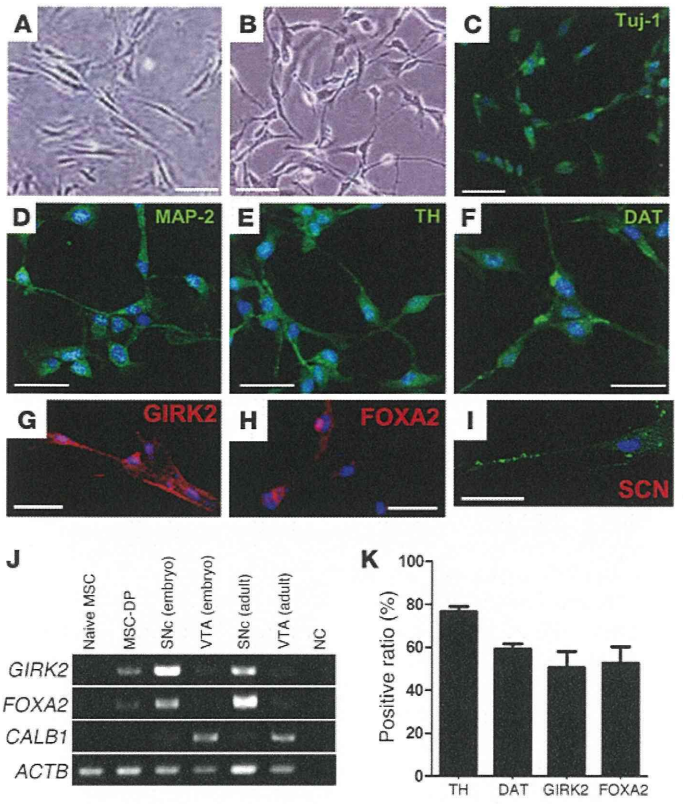

Figure 1
Monkey bone marrow MSCs and MSC-DP cells. (**A**) Morphological changes were evident in cynomolgus naive MSCs (phase-contrast microscopy) and (**B**) MSC-DP cells (phase-contrast microscopy). The MSC-DP cells possessed neurite-like processes. Immunocytochemistry of MSC-DP cells showed that the cells were immunoreactive for the neuronal markers (**C**) Tuj1 and (**D**) MAP-2; for the markers of dopaminergic neurons (**E**) TH, (**F**) DAT, (**G**) GIRK2, and (**H**) FOXA2; and for the marker of neurons, (**I**) sodium channel (SCN). DAPI was used for counterstaining of nuclei. Scale bar: 30 μm. (**J**) Results of RT-PCR in naive MSCs, MSC-DP cells, and tissue samples from the SNc and VTA. Naive MSCs expressed no *GIRK2*, *FOXA2*, and *CALB1* mRNA, while the MSC-DP cells expressed *GIRK2* and *FOXA2* mRNA. SNc tissue samples from an embryo and an adult cynomolgus monkey also contained high levels of *GIRK2* and *FOXA2* mRNA but only low levels of *CALB1* mRNA, while the VTA contained high levels of *CALB1* mRNA and only very low levels of *GIRK2* and *FOXA2* mRNA. NC, negative control. (**K**) Percentages of cells immunoreactive for TH, DAT, GIRK2, and FOXA2 in MSC-DP cells.

each test animal, expressed cell makers not only for antigens that have been previously described (20, 21), such as β tubulin III (Tuj1), microtubule-associated protein 2 (MAP-2), TH, and DAT, but also for those specific to the A9 subtype, namely, G protein–coupled inward rectifying current potassium channel type 2 (GIRK2) (15) and forkhead box protein A2 (FOXA2) (28). The effect of transplantation was evaluated for up to 9 months based on motor behaviors of affected hand movements; PET scans using ^{11}C-CFT, which specifically labels DAT; and postmortem histology. Tumorigenicity was also estimated from the results of blood tests and PET scans. The preclinical data obtained thus may extend the applicability of the current autologous cell system as a therapy for PD.

Results

Evaluation of MSC-DP cells. Cynomolgus monkey MSCs drastically changed their morphology following induction, as reported previously (21): naive MSCs initially showed fibroblast-like mesenchymal cell features (Figure 1A), while induced cells showed a neuron-like morphology with neurite-like processes (Figure 1B). By immunohistochemistry, naive MSCs were negative for neuronal markers, as reported previously (21), but induced cells were positive for neuronal markers, Tuj1 (Figure 1C) and MAP-2 (Figure 1D); dopaminergic neuron markers, TH and DAT (Figure 1, E and F); the A9 dopaminergic neuron marker, GIRK2 (Figure 1G); and a marker of floor plate–derived cells, FOXA2 (Figure 1H). We also confirmed that these cells were positive for sodium channels (Figure 1I), a marker of differentiated neurons.

To confirm whether these cells have an ability to produce and release dopamine, we measured the secretion of dopamine by HPLC. The amount of dopamine in the culture supernatant was measured following application of high K^+ depolarizing stimuli, which resulted in release of 1.04 ± 0.4 pM dopamine per 10^6 cells (Table 1); by contrast, naive cynomolgus monkey MSCs showed no detectable dopamine release. These results are consistent with those of our previous studies: the amount of dopamine release was comparable to the amounts in rats (1.1 pM/10^6 cells) (20) and monkeys (0.9 ± 0.2 pM/10^6 cells) (21).

We further investigated the expression of markers specific for A9 dopamine neurons using RT-PCR. The MSC-DP cells expressed *GIRK2* and *FOXA2* mRNA but not *CALB1* mRNA (*GIRK2*$^+$/*FOXA2*$^+$/*CALB1*$^-$) (Figure 1J). When control tissues obtained from a cynomolgus embryo and an adult animal were analyzed, both showed that the substantia nigra pars compacta (SNc) was strongly positive for GIRK2 and FOXA2 and weakly positive for calbindin, while the ventral tegmental area (VTA) was weakly positive for GIRK2 and FOXA2 but strongly positive for calbindin (Figure 1J). This distinct pattern of GIRK2/FOXA2/calbindin expression in the SNc and VTA is consistent with those reported in other species, including rodents and humans (15, 28, 29). We also evaluated the induction efficiency of MSC-DP cells by quantitative immunocytochemistry. Fifty to seventy-five percent of MSC-DP cells were positive for TH, DAT, GIRK2, and FOXA2 (Figure 1K). These findings indicated that the current method efficiently produced MSC-DP cells from the MSCs, which had properties similar to those of the A9 dopamine neurons in the model species.

Behavioral analysis of motor symptoms. The clinical rating scores (CRSs) for parkinsonian animals are shown in Figure 2A. The CRS revealed a significant interaction effect between groups and time

Table 1
Dopamine-producing capacity of MSC-DP cells and the number of engrafted cells

Animal ID	Dopamine release induced by K^+ (pM/10^6 cells)	No. of engrafted cells (× 10^6 counts)
mon0703	2.6	20.4
mon0705	0.5	9.0
mon0708	0.4	12.0
mon0709	0.8	12.7
mon0710	0.9	18.6

Dopamine release induced by K^+ was measured by HPLC. Animals were from the MSC-DP–engrafted group.

after transplantation (F distribution [$F_{4,32}$] = 3.07, $P < 0.05$). Thus, we further tested for an effect of time, separately in each of the groups in our study (engrafted or sham), by 1-way ANOVA. The CRS in the MSC-DP–engrafted group showed a marginal effect of time ($F_{4,16} = 2.95$, $P = 0.055$). In a post-hoc comparison of scores at each time point after engraftment with those at baseline, significant improvements in the CRS were observed at 8 months after engraftment (Dunnett's multiple comparison, $P < 0.05$). This time effect was not observed in the sham group either by 1-way ANOVA or post-hoc analysis.

Hand-reach scores showed similar time courses (Figure 2B). The scores for the affected hand showed significant group and time interaction ($F_{4,32} = 2.83$, $P < 0.05$). One-way ANOVA for the repeated measures of hand-reach scores for the affected hand of the MSC-DP–engrafted group revealed a significant effect of time ($F_{4,16} = 5.62$, $P < 0.001$). A post-hoc comparison of scores at each time point after engraftment with those at baseline revealed significant improvements in hand-reach scores at 4 months (Dunnett's multiple comparison, $P < 0.05$) and 8 months ($P < 0.01$) after engraftment. This effect of time was not observed in the sham group. Therefore, the CRSs and hand-reach scores suggested that the engraftment of MSC-DP cells modestly improved motor behaviors in parkinsonian animals.

Despite these improvements in MSC-DP–grafted animals, spontaneous activities of animals were not affected by any of group (sham vs. MSC-DP engrafted), time (before engraftment and 4 months and 8 months after engraftment), or interaction among these variables (2-way ANOVA with repeated measures, Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI62516DS1). Animals in the engrafted group tended to show higher spontaneous activities than sham-operated animals at 4 and 8 months after engraftment; however, no statistically significant effect of group was observed at any time point in post-hoc analysis (Bonferroni corrected, $P > 0.05$). Within-subject and between-subject data were highly variable, consistent with a previous report in this type of parkinsonian animal model (30). No dyskinesia-like abnormal movements were observed in any MSC-DP cell–engrafted animals during the observation period.

^{11}C-CFT PET. Voxel-based analysis of ^{11}C-CFT binding potential (BP_{ND}) images disclosed a significant effect of time in a cluster extending into the dorsal posterior putamen in the engrafted group (Figure 3C) (family-wise error rate [FWE] corrected, $P < 0.05$; Table 2). The maximum of this cluster was located ($x = 11.5$ mm, $y = -8.0$ mm, $z = 3.5$ mm) in a standard macaque brain space of the Montreal Neurological Institute (MNI) (31) and was safely within the area targeted when engrafting MSC-DP cells into the striatum (Figure 3A). Using this cluster as a region of interest (ROI), we obtained BP_{ND} values for this ROI across all animals, groups, and time points. The BP_{ND} values obtained are presented in Figure 3D. Two-way ANOVA with repeated measures (Figure 3D) revealed significant effects of an interaction between group and time ($F_{4,24} = 4.3$, $P < 0.01$). Post-hoc analysis showed that the BP_{ND} at 7 days after engraftment was higher than that in the sham-operated group (Bonferroni multiple comparison, $T = 4.56$, $P < 0.001$), as shown in Figure 3D. In particular, animal mon0703, who received the graft with the largest amount of MSC-DP cells (Table 1), showed the highest BP_{ND} (0.59) in the engrafted striatum at 7 days after engraftment, followed by animal mon0710, who showed a BP_{ND} of 0.43 at 7 days after engraftment (Figure 3B).

The time-dependent decline in ^{11}C-CFT binding in the engrafted striatum (Figure 3D) allowed us to analyze the kinetics of ^{11}C-CFT binding in detail. Because recent studies have suggested that MSCs are susceptible to senescent (12) and apoptotic changes (13), we supposed that this decline was due to degeneration of engrafted MSC-DP cells. ^{11}C-CFT binding is known to be correlated with the density of dopamine neurons or terminals rather than any physiological (or functional) variation in dopamine release. The rate of ^{11}C-CFT binding reduction, calculated based on the 1-hit model of neurodegeneration (32), was 0.30 months (~10 days) as a half-life period (Figure 3E). This rate of reduction was slightly slower than that for engrafted naive rodent MSCs (~3 days; Supplemental Figure 2) based on previously described data (13). We further tested whether this degenerative process would affect all grafted cells. The plateau of the 1-hit model was significantly higher than the baseline (before engraftment) ^{11}CFT binding level (baseline $BP_{ND} = 0.087 \pm 0.028$ vs. BP_{ND} at plateau $= 0.17 \pm 0.029$; Welch's corrected $T_{13} = 2.04$, 1-tailed $P < 0.05$), suggesting that a small portion of the grafted MSC-DP cells survive and integrate

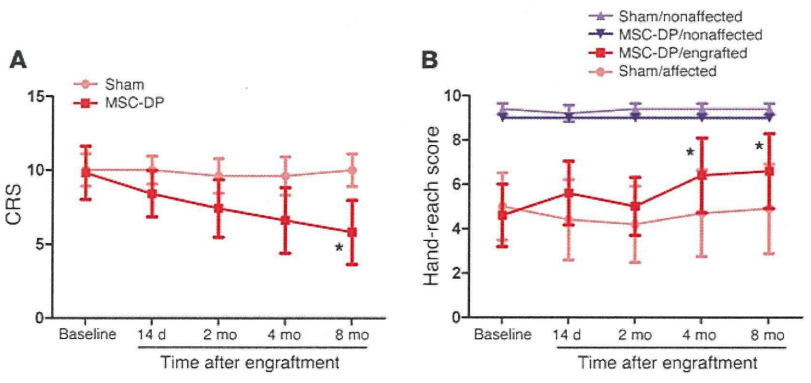

Figure 2
Behavioral assessment. (**A**) CRSs and (**B**) hand-reach scores were plotted against time for MSC-DP cell–engrafted (MSC-DP) and sham-operated (Sham) groups. *$P < 0.05$ compared with baseline in the MSC-DP–engrafted group, Dunnett's multiple comparison test.

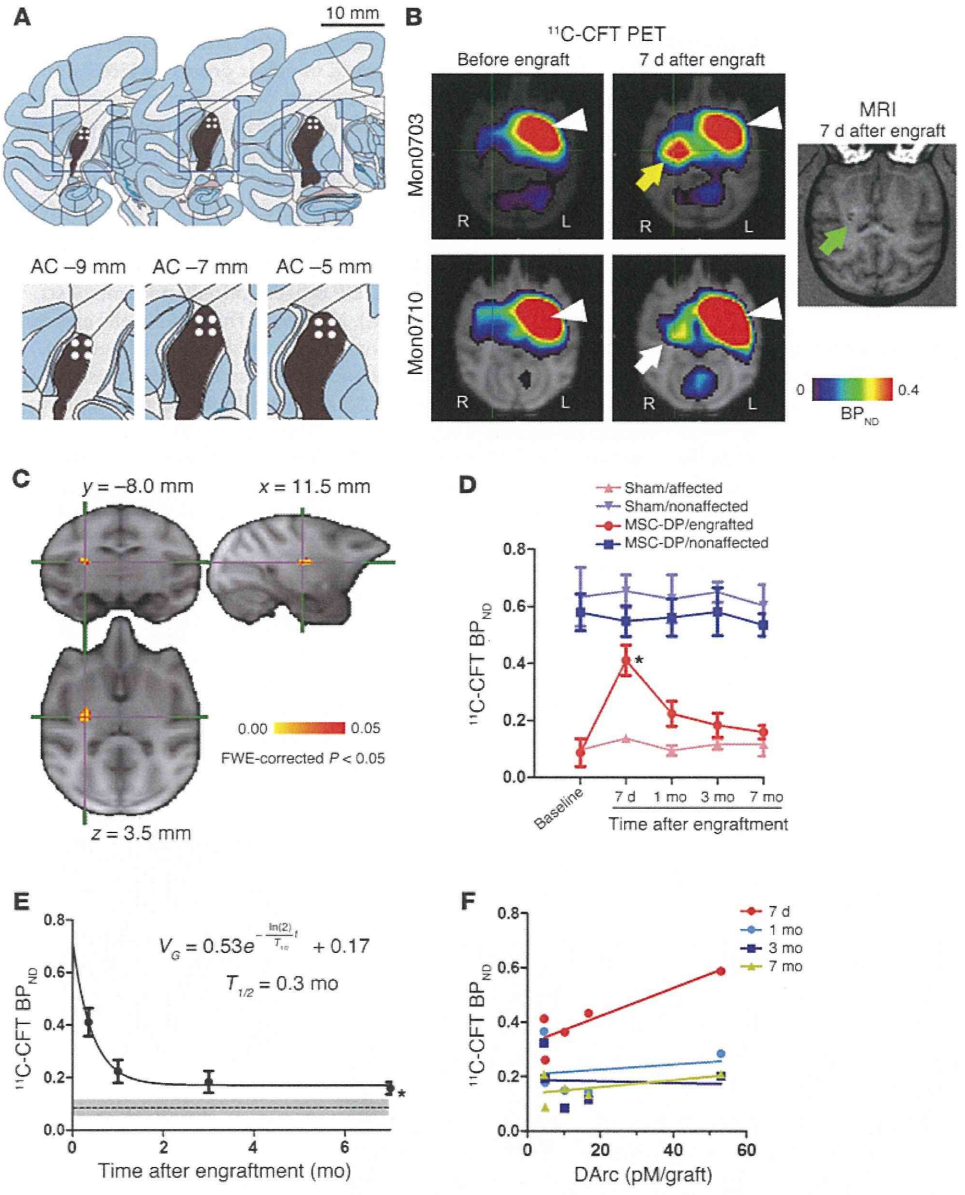

Figure 3

Engrafting and neuroimaging of MSC-DP–engrafted animals. (**A**) Twelve target points (white dots) for engraftment in putamen in the coronal sections of standard space of cynomolgus macaque. Scale bar: 10 mm. (**B**) Representative 11C-CFT PET images of MSC-DP–engrafted animals. Seven days after engraftment (engraft), animal mon0703 showed the highest 11C-CFT BP_{ND} on the MPTP-treated side of striatum (yellow arrow), followed by animal mon0710 (white arrow). Animal mon0710 showed low signal (green arrow) in a T1-weighted MRI after engraftment, which diminished in later scans. Note that the BP_{ND} in the non-MPTP–treated (and nonengrafted) side of striatum was very high (white arrowheads) and not different by engraftments. (**C**) Significant effect of time on 11C-CFT BP_{ND} (P values range from yellow [$P < 0.00$] to red [$P < 0.05$]) overlaid on the study-specific MRI template in MNI space. A cluster with FWE-corrected $P < 0.05$ was located in the dorsal posterior putamen in the engrafted striatum. See also Table 2. (**D**) Time course of 11C-CFT BP_{ND} in the cluster (in **C**) and contralateral equivalent region. *$P < 0.05$ compared with sham, Bonferroni corrected. (**E**) Time course of 11C-CFT BP_{ND} values in the cluster (in **C**), fitted by the 1-hit (exponential) model of neurodegeneration. The dashed line and gray area indicate the mean and SEM for the baseline BP_{ND}. *$P < 0.05$ between the baseline BP_{ND} and the plateau of the fitted model. (**F**) The DArc and the 11C-CFT BP_{ND} values at 7 days and 1, 3, and 7 months after engraftment.

for more than 7 months. This is in contrast to findings in naive MSCs engrafted into rodents, which showed a plateau of the reduction curve returned to the baseline (Supplemental Figure 2), indicating that all naive MSCs may eventually degenerate when transplanted into brain.

We also estimated how the dopamine-releasing capacity (DArc) of grafts related to 11C-CFT binding after engraftment (Figure 3F). The dopamine-releasing capacity was calculated by multiplication of dopamine release by K^+ concentration (pM per 10^6 cells) in vitro, as measured by HPLC, and the total number of cells engrafted in each animal (10^6 cells). This graft DArc (pM/graft) significantly predicted the 11C-CFT BP_{ND} obtained from the cluster of engrafted striatum scanned at 7 days after engraftment (linear regression, $BP_{ND} = 5.1 \times 10^{-3} \times \text{DArc} + 0.31$, $F_{1,3} = 10.3$, $P < 0.05$; Figure 3F) but not those obtained at later time points. Although dopamine release and DAT expression are different measures, both are a common and specific attribute of well-differentiated dopamine neurons (33). Therefore, the findings indicate that the DAT expression at the earliest time point after transplantation is coupled to the dopaminergic capacity of the engrafted cells, while other factors may have degraded this coupling at later time points.

Tests for tumorigenicity and general condition of animals with MSC-DP cell engraftment. There were no abnormal changes in the results of blood tests, including red and white blood cell counts, and blood chemical tests in any of the MSC-DP– and sham-treated animals tested (Supplemental Table 1). There was also no abnormal

Table 2
Voxel-based statistical results of ^{11}C-CFT BP_{ND} in repeated-measure ANOVA

Cluster	No. of voxels	Maximum voxel			
		x (mm)	*y* (mm)	*z* (mm)	F value
Right dorsal posterior striatum	224	11.5	–8.0	3.5	14.6

The location of the maximal voxel is presented in *x*, *y*, and *z* coordinates of the standard anterior-posterior commissural coordinates in MNI space. See Figure 3C for visual presentation of this cluster. FWE-corrected $P < 0.05$.

elevation in the levels of the tumor markers carcinoembryonic antigen (CEA), tissue polypeptide antigen (TPA), sialyl Lewis X antigen (SLX), neuron-specific enolase (NSE), and basic fetoprotein (BFP) (Supplemental Table 2).

^{18}F-FDG PET scans revealed normal uptake of ^{18}F-FDG in the engrafted striatal region of animals in the engrafted group (Figure 4). In MRI scans, 2 animals (mon0710 and mon0049) showed subtle hemorrhagic changes, as suggested by low signals in the engrafted region of the right striatum in T1-weighted MRI images at 7 days after engraftment (Figure 3B); however, no significant signal changes were observed in later MRI scans. Therefore, no enlargement of or tumor formation by the engrafted tissues was suspected, at least at the macroscopic level, up to 8 months after transplantation of MSC-DP cells. These findings were confirmed by histological evaluations, as described below.

Immunohistochemical study. Consistent with the ^{8}F-FDG PET scans and MRI data, no tumor formation was observed in either group by H&E staining (data not shown). We further evaluated the proliferative activity of MSC-DP cells engrafted into the striatum by immunostaining using a Ki-67 antibody. Although, we found a small number of Ki-67$^+$ cells in the MSC-DP cell–engrafted striatum (Figure 4B), the proportion of Ki-67$^+$ cells was <2.5%, and there was no significant difference between MSC-DP cell–engrafted and control striata (Figure 4C). Moreover, the Ki-67$^+$ cells existed solitarily, and none of them formed tumor-like masses (Figure 4B).

We then performed triple staining of striatal sections using markers, including those for A9 and A10 dopaminergic neurons. In the transplanted region of MSC-DP cell–engrafted striata, cells positive for DAT and GIRK2 but not for calbindin were clearly observed (Figure 5, A–F), but such cells were not found in any of sham-operated striata. The staining pattern of A9-type dopaminergic neurons was confirmed by analyzing the midbrain of a normal control adult animal with the same triple staining (Supplemental Figure 3): the A9-type dopaminergic neurons in the SNc showed a pattern of DAPI$^+$/DAT$^+$/GIRK2$^+$/calbindin$^-$ (Supplemental Figure 3A), while the A10-type neurons in the VTA had a pattern of DAPI$^+$/DAT$^+$/GIRK2$^-$/calbindin$^+$ (Supplemental Figure 3B). We also confirmed that most of the DAT$^+$ cells in the engrafted striata were positive for TH (Figure 5, G–J) but not for a glial marker, glial

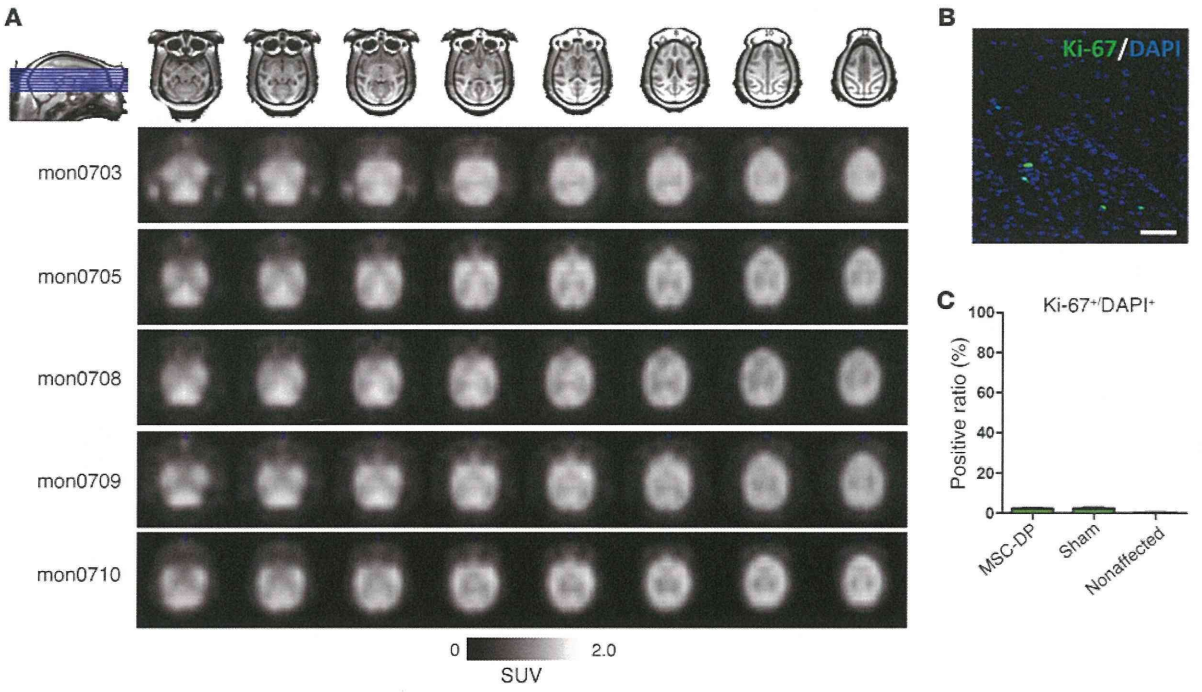

Figure 4
Evaluation of tumorigenicity of MSC-DP–grafted animals. (**A**) SUV images of ^{18}F-FDG PET scans, obtained 8 months after engraftment, are shown for each animal in the engrafted group. No apparently high uptake of ^{18}F-FDG was found in any of MSC-DP cell–engrafted animals. (**B**) Immunostaining with Ki-67 is shown in a section of MSC-DP cell–engrafted striatum. Cells positive for Ki-67 (green) scarcely existed in the dorsal-posterior putamen. Scale bar: 100 μm. (**C**) Quantitative analysis of the ratio of Ki-67$^+$ cells to the total number of cells in 3 groups of striatum (MSC-DP grafted, sham operated, nonaffected). See also Supplemental Tables 1 and 2 for the results of blood tests for biochemical and tumor markers.

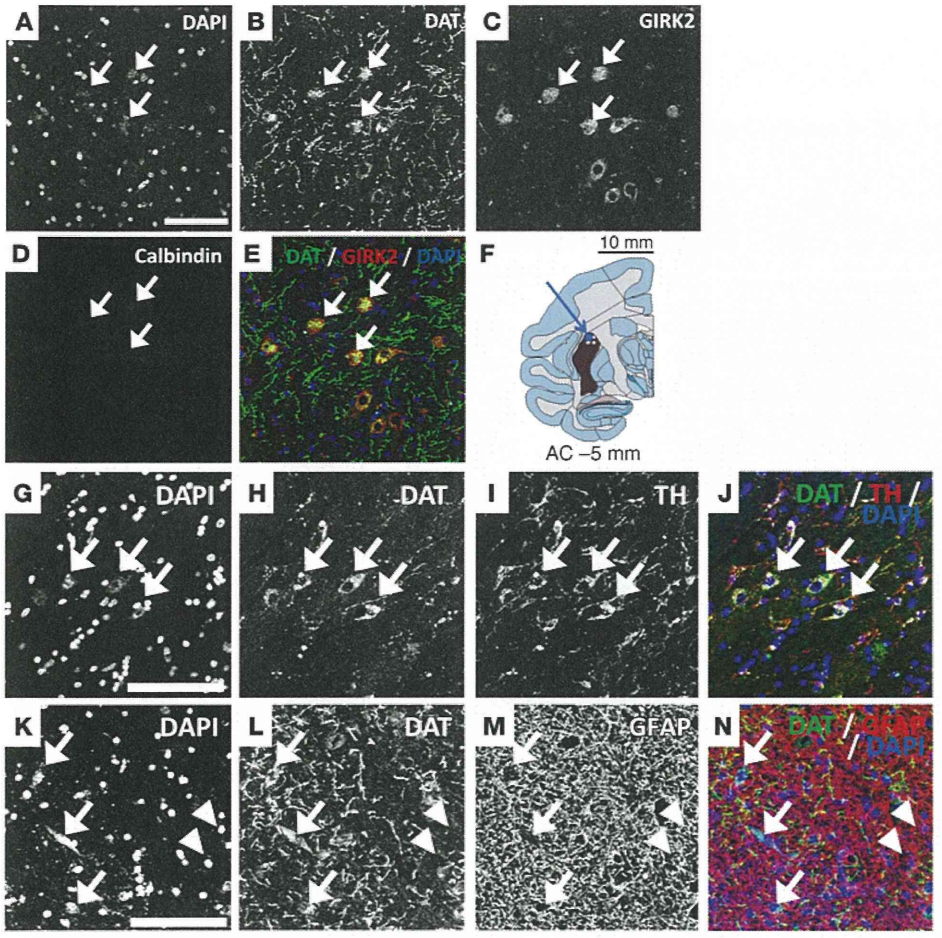

Figure 5
Immunohistochemistry of the MSC-DP–engrafted striatum. In the MSC-DP–grafted striatum, the cell bodies positive for DAT and GIRK2 (arrows in **B** and **C**, respectively) but not for calbindin (arrow in **D**) were found in the area close to the MSC-DP cell–engrafted area (white dots in **F**) in the dorsal posterior putamen. (**A**) DAPI was used for counterstaining of nuclei. (**E**) A merged image for DAT, GIRK2, and DAPI is shown in green, red, and blue, respectively. Most of the DAT^+ cells were also positive for TH (arrows in **G**–**J**) but not for GFAP (arrows in **K**–**N**), while the $GFAP^+$ cells with round-shaped nuclei, putative astroglial cells, were not positive for DAT (arrowheads in **K**–**N**). Scale bar: 100 μm (**A**–**E** and **G**–**N**); 100 mm (**F**).

fibrillary acidic protein (GFAP) (Figure 5, K–N). Taken together, these findings indicate that the transplanted MSC-DP cells survived and maintained the characteristics of A9-type dopaminergic neurons for at least 9 months after transplantation.

Quantitative analysis revealed that the number of cells positive for NeuN tended to be higher in the MSC-DP–engrafted striata than in sham and nonaffected (i.e., non-1-methyl-4-phenyl-1,2,3,6-tetrahydoropyridine–treated [non-MPTP–treated]) striata, but no statistically significant differences were found among the 3 groups of striata ($F_{3,112} = 1.12$, $P > 0.05$; Figure 6, A and B). The number of TH^+ cell bodies in the engrafted region was higher in the MSC-DP group than in the sham and nonaffected groups, with statistical significance ($F_{2,88} = 5.99$, $P < 0.005$, Tukey's multiple comparison $P < 0.05$; Figure 6, A and C). In contrast, the number of TH^+ axon terminals was higher in the MSC-DP group than in the sham group, with statistical significance ($F_{2,119} = 3.93$, $P < 0.05$, Tukey's multiple comparison $P < 0.05$; Figure 6, A and D), suggesting that the TH^+ cell bodies observed in the engrafted striatal group integrated with host striatal neurons by forming new synaptic connections. Similarly, the number of DAT^+ cells was also higher in the MSC-DP group than in the sham and non-affected striatum groups, with statistical significance ($F_{2,74} = 16.78$, $P < 0.0001$, Tukey's multiple comparison $P < 0.0001$; Figure 6, A and E).

Discussion

Early transplantation trials for PD treatments used fetal midbrain tissue as a cell source. Successful results were first reported in studies using macaques in the late 1980s (22–24). Subsequent open-label trials in patients with PD also showed that engrafted fetal neurons can survive, appropriately differentiate, and provide striatal dopamine release as measured by PET and showed clinical improvements (34–36), with only one exception (37). Although later double-blind placebo-controlled trials reported no significant clinical benefits (38, 39), successful cell replacement therapy in PD would probably be achieved with more sophisticated cell preparation, surgical and patient selection procedures (2, 3). In particular, optimization of cell preparation has been an issue when using fetal tissue because of its limited availability and accessibility; thus, there is an urgent need to develop alternative cell sources (40). In addition, recent basic studies based on fetal tissue graft systems have suggested several potential refinements. For example, whether or not the graft contains A9 dopaminergic neurons may be a determinant factor for achieving synaptic formation with host tissues and better behavioral recovery (16). Others have also suggested that expression of DATs in the graft cells alleviates dyskinesias after graft (41). Therefore, proof-of-concept studies using fetal tissue grafts have established the direction of cell-based therapies, including those using stem cells (2, 3, 40). Particular concerns when

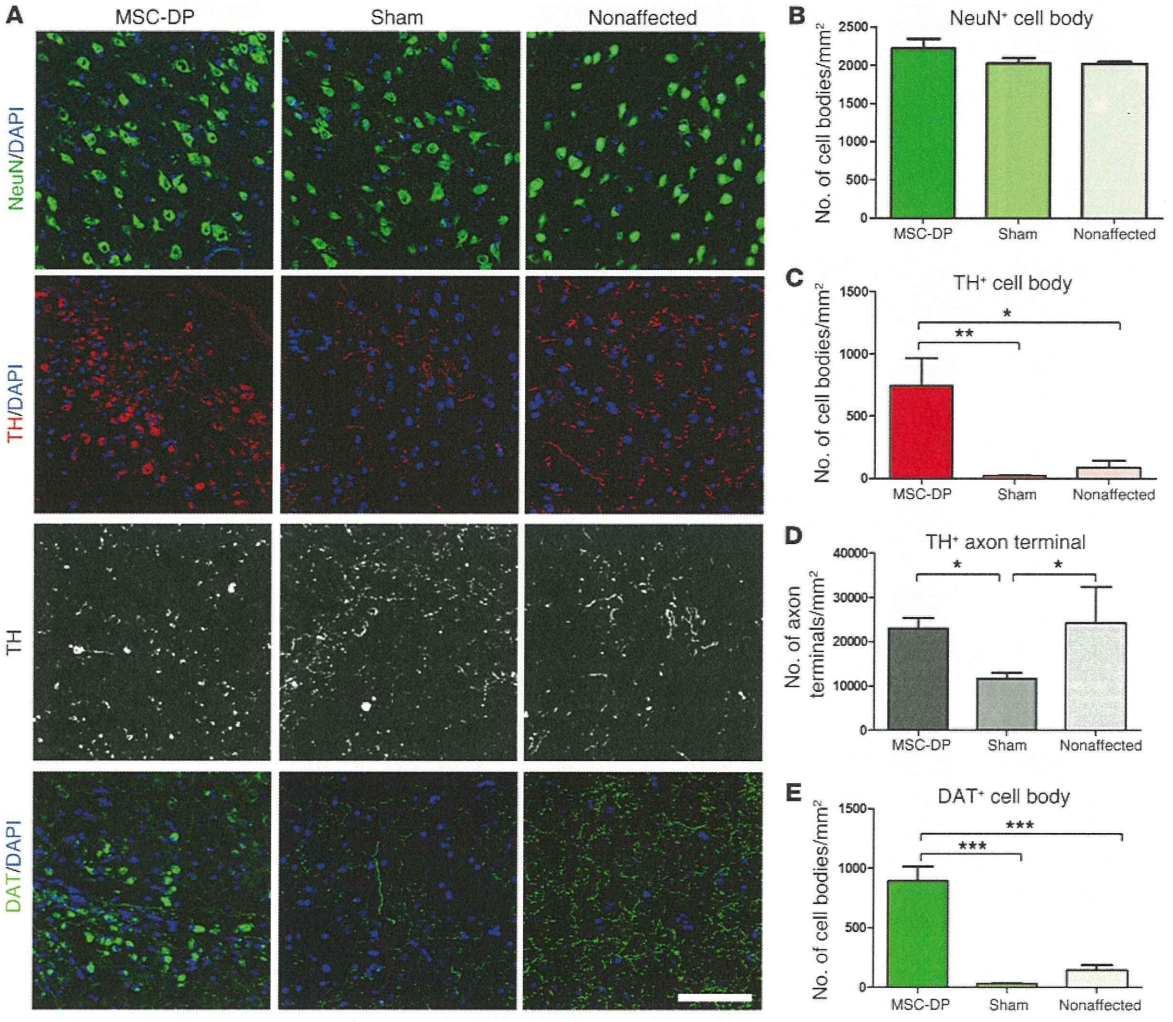

Figure 6

Quantitative analysis of immunohistochemistry of the dorsal posterior striatum. (**A**) Immunohistochemical sections of the dorsal posterior striatum for NeuN, TH$^+$ cells, TH$^+$ terminals, and DAT$^+$ cells in MSC-DP cell–engrafted animals (MSC-DP), in the sham-operated striatum (Sham), and in the non-MPTP–treated side of the striatum (nonaffected) of sham animals. Scale bar: 100 μm. (**B**) Cell counts of NeuN$^+$ and (**C**) TH$^+$ neuronal cell bodies, (**D**) TH$^+$ axon terminals, and (**E**) DAT$^+$ neurons in the dorsal posterior striatum were plotted for each group. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, Bonferroni-corrected multiple comparisons.

applying stem cells are functionality and safety issues after graft, both of which need to be overcome before clinical trials can be commenced (40). From these standpoints, we examined whether the MSC-DP cells could safely function in primate PD model.

Our results can be summarized into the four points. First, the MSC-DP cells induced from macaque MSCs were confirmed to express markers for Tuj1, MAP-2, TH, and DAT and to release dopamine in vitro, as shown previously (21). Moreover, they expressed GIRK2 and FOXA2, specific markers of A9 dopaminergic neurons, but not calbindin, a marker of A10 neurons. Second, when MSC-DP cells were autologously engrafted into the striatum of hemiparkinsonian macaques, motor behaviors, as assessed by CRS and the hand-reach task, gradually improved without accompanying dyskinesia. PET scans detected distinct binding of ^{11}C-CFT in the engrafted striatum initially, followed by an exponential decrease in ^{11}C-CFT binding. Kinetic analysis suggested that a portion of the MSC-DP graft showed levels of ^{11}C-CFT binding above the baseline over a period of 7 months. Third, immunohistochemical analysis at 9 months confirmed the increase of DAT$^+$/TH$^+$/GIRK2$^+$ cells and the TH$^+$ axon terminals in the engrafted striatum. Fourth, no tumorigenicity was suspected by peripheral blood tests, blood tumor markers, ^{18}F-FDG PET scans, and histology. Therefore, MSC-DP cells are likely to be nontumorigenic and able to remain in the transplanted region until at least 9 months after engraftment, although not in large quantities, thereby modestly restoring dopaminergic function and motor behaviors in vivo.

To the best of our knowledge, this study is the first to show restoration of dopaminergic function and motor behaviors in parkinsonian primate animals following engraftment of MSC-de-

rived cells. It is notable that kinetic analysis of ^{11}C-CFT binding and histology results suggested that at least a portion of MSC-DP cells survived in situ for over 9 months. This is in contrast to naive MSCs, which have much shorter survival times in the brain and disappeared within 3 months in rodent studies; one possible reason for this could be senescent or apoptotic changes (12, 13). The long-term survival of MSC-DP cells may have been accompanied by gradual integration into or reinnervation of the host striatal tissues. As shown in Figure 6D, increased numbers of TH$^+$ terminals were observed in engrafted animals compared with control animals, suggesting formation of new synaptic connections between the engrafted cells and host tissues; this may at least in part underlie the motor recovery observed at a later time after transplantation of MSC-DP cells. The fact that in vitro DArc was correlated with ^{11}C-CFT binding only at early time points after engraftment also supports the hypothesis that other mechanisms, such as integration of MSC-DP cells, into host tissues may be critical for full restoration of motor function.

A notable feature of the current MSC-DP cells is their similarity to A9 dopaminergic neurons. Previously, we reported that dopaminergic neuron-like cells are inducible from MSCs by genetic introduction of *NICD*, followed by cytokine treatment with bFGF, forskolin, CNTF, and GDNF (20, 21). This report further characterized the MSC-DP cells and found that they were double positive for FOXA2 and GIRK2, both specific markers of differentiated A9 dopaminergic neurons. In particular, FOXA2 was recently identified as a transcription factor required for specifying and maintaining the dopaminergic neuron phenotype (28, 42) and is considered to be expressed exclusively in floor plate–derived dopaminergic neurons (43). We confirmed the coexpression of FOXA2 and GIRK2 in SNc tissue that contains A9 dopaminergic neurons but not in the VTA, which contains A10 dopaminergic neurons. GIRK2/FOXA2 double-positive cells have not been induced from other stem cell sources using existing protocols (44–46), except for one that manipulates both the sonic hedgehog and WNT signaling pathways (43, 47). Recent studies of fetal tissue engrafting in patients with PD or PD model rats have shown that A9 dopaminergic neurons are determinants of successful functional recovery (16, 48) and decreased dyskinesia (49) and that they are associated with well-organized synaptic connectivity (16). Overall, the results of our study support a recent hypothesis that well-differentiated stem cells offer benefits for both survival and functional integration with host neural tissues (47).

There are 2 points that favor the suitability of MSC-DP cell transplantation for clinical application. First, the current strategy rests on the autologous cell system. This offers advantages over allograft systems, such as fetal tissue engrafting, in terms of the ethical, social, and political implications. MSCs can be easily collected autologously from the patients' own bone marrow aspirates. Aspiration of bone marrow by itself is widely applied in the field of hematology, and infusion of bone marrow from HLA-matched donors is also commonly performed for treatment of leukemia. Moreover, bone marrow–derived MSC transplantation into brain has been tried clinically without any safety problems (10). The autologous system also has the advantage of not requiring immunosuppression, as compared with allograft systems. Second, no tumorigenicity was suspected up to 9 months after engraftment in primates. Adult stem cells, including MSCs, are known to have less tumorigenic proliferative activity than other cell sources, such as embryonic stem cells. Moreover, the MSC-DP cells expressed MAP-2, a marker of postmitotic neurons (50), suggesting that they possess minimal risk of tumor formation. A previous study in which MSC-DP cells were engrafted into rodents consistently showed no evidence of tumorigenicity during a 14-week period (20). Schwann cells derived from MSCs also showed no tumorigenicity during a 1-year observation period in our previous study in primates (18). The differentiation technique for converting naive MSCs to MSC-DP cells involved *NICD* gene introduction by transfection of a plasmid gene but not by retrovirus- or lentivirus-mediated gene transfer, which is known to modify the nuclear genome. The introduced plasmid *NICD* gene does not appear to have a prolonged effect on the cell cycle, because the introduced *NICD* gene did not remain in MSCs for a long time (for no longer than 2 passages) (51).

Although our study showed a potential therapeutic effect of MSC-DP cells in a PD model, one may argue that cells pushed to full maturity in vitro would not be the best cell source for grafting (2). For example, grafted dopaminergic neurons obtained from fetal brain are known to survive poorly if their maturation has passed a narrow optimal time window at the time of harvesting (52). Such an argument might be applicable to our results in that the rapid loss of ^{11}C-CFT binding over time may have been a consequence of the use of differentiated MSCs, namely MSC-DP cells. However, this is still an open question, particularly with respect to stem cells; the relationship between in vitro maturity and survival after graft is not simple and may also depend on other factors, such as the types of cell sources and in vitro preparation. ES grafts with very immature cells seem to cause tumorigenicity, while relatively immature cells, such as neural precursors/neuroblasts, suffer from poor cell survival (40). But more highly differentiated DA neurons recently engineered from ES cells showed good survival (47). Undifferentiated MSCs are known to die very rapidly, within several days after being engrafted into the brain, and this seems to be due to facilitated senescence (12) or programmed cell death of the MSCs (13). Therefore, we have followed the working hypothesis, testing well-differentiated cells derived from MSCs. To address the issue of optimal maturity, cell marker–based subtyping should also be standardized in future studies (33).

A few issues suggest the need for some caution when interpreting our results. First, in this study, we did not label the MSC-DP cells by tagging them with, for example, green fluorescent protein in order to trace the cells after transplantation. This was because we avoided using any extrinsic factors that may pose a safety concern or affect cell survival. As a result, the dopaminergic neuron-like cells found in the striatum may not truly have originated from the grafted MSC-DP cells. However, this is unlikely because the striatum in primates has only a few dopaminergic neurons under normal circumstances: TH$^+$ cells number <1% of striatal neurons (53) and are preferentially scattered near the white matter–ventral striatum border. In our case, the TH$^+$/DAT$^+$/GIRK2$^+$ cells were located only in an area near the engrafted area in the dorsal striatum (Figure 5), and the number of the cells was larger than that in sham and nonaffected striata (Figure 6, C and E). Second, regarding PET ligands, a well-known ligand for DA, ^{18}F-DOPA was not used in this study, because it is a metabolic ligand for amino acids and may reflect not only dopamine turnover in dopaminergic neurons, but also uptake of amino acid precursors in tumors (54) or inflammation (55). Therefore, ^{11}C-CFT was carefully chosen as an in vivo surrogate marker for tracking MSC-DP cells, because it is a highly specific ligand for DAT, which is specif-