Autologous transplantation of GDNF-expressing mesenchymal stem cells protects against MPTP-induced damage in cynomolgus monkeys

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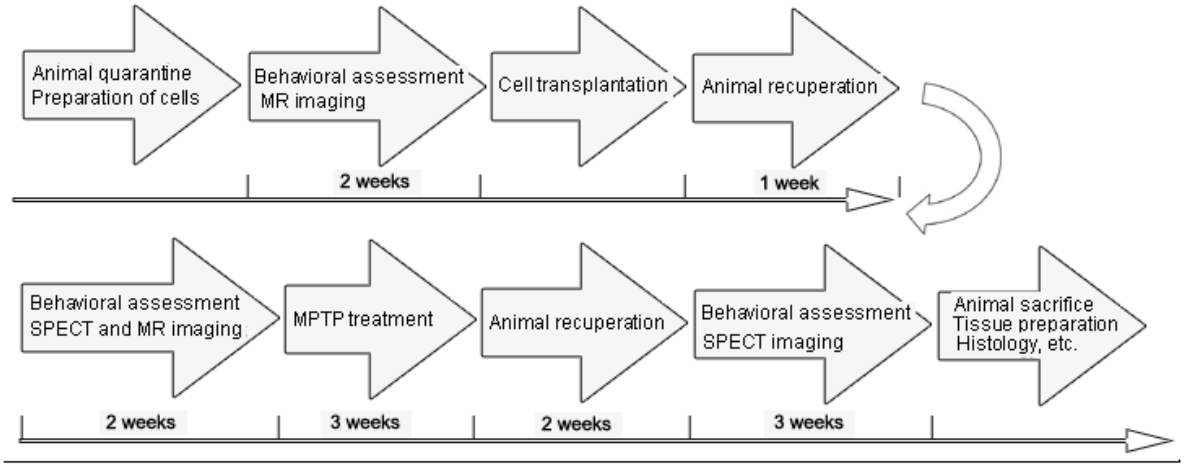
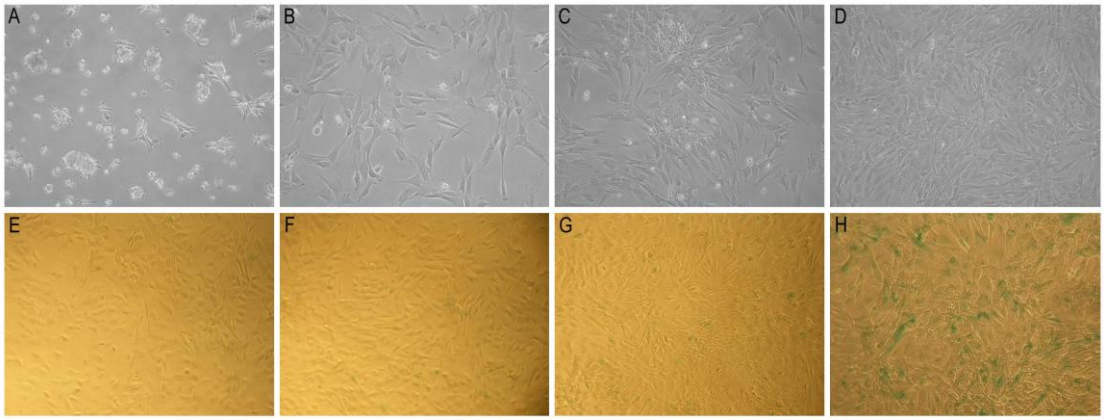
Supplementary information

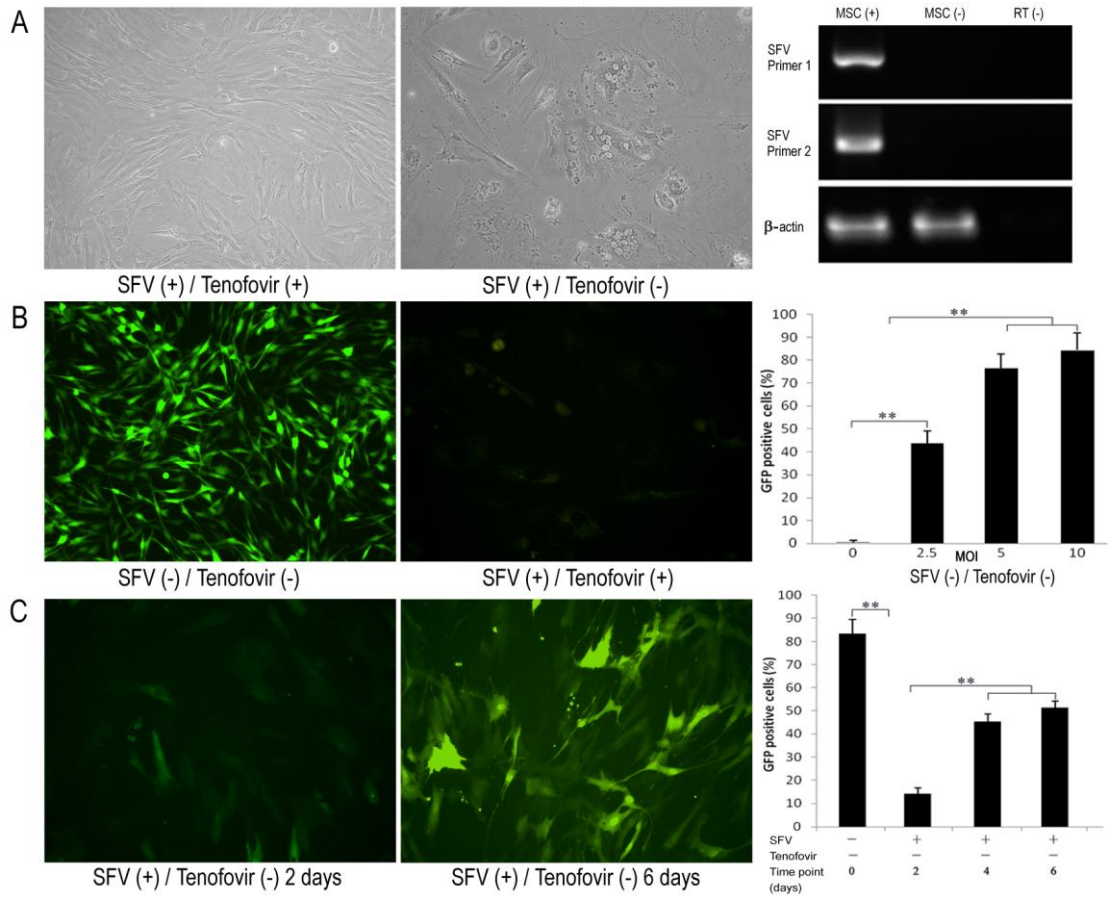


Supplementary Figure 1. Schematic diagram to illustrate the experimental paradigm.



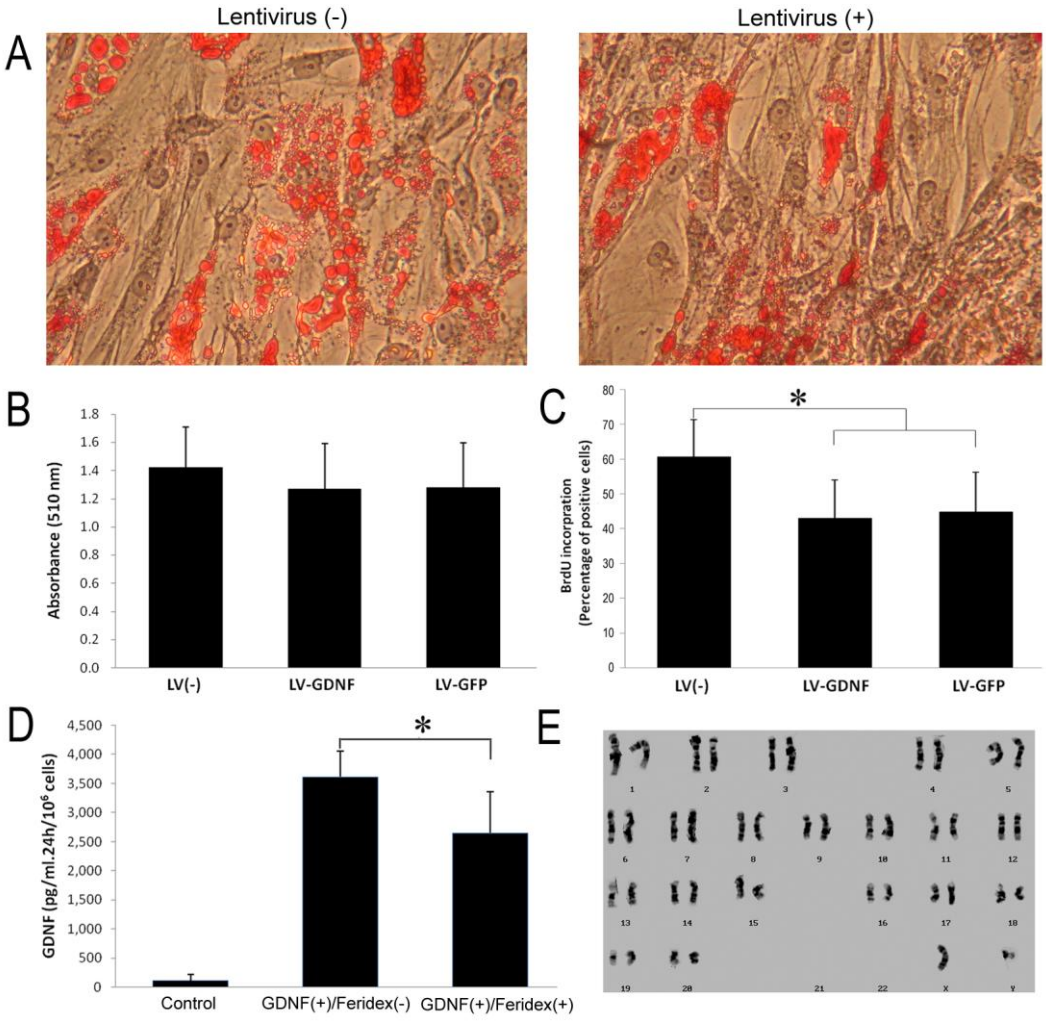
Supplementary Figure 2. Culture of MSCs.

MSCs were isolated from the bone marrow of individual cynomolgus monkeys. (A-D) primary MSC cultures on day 2 (A), 4 (B), 6 (C), and 8 (D) in vitro; (E-H) β-galactosidase activity of MSCs at passage number 2 (E), 4 (F), 8 (G) and 12 (H). (A-H) 200×.



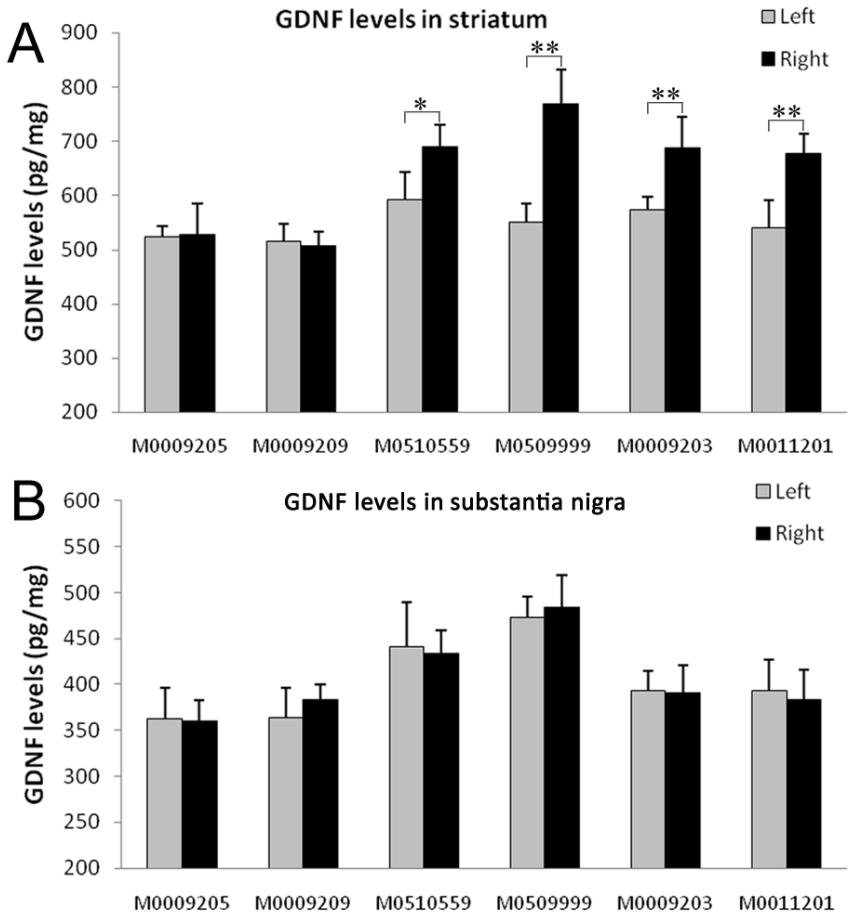
Supplementary Figure 3. Lentivirus infection of SFV+ MSCs requires temporal removal of Tenfovir.

(A) The antiviral drug Tenofovir had to be added to maintain proper culture of MSCs. Left panel, SFV-positive culture with Tenofovir treatment; Middle panel, without Tenofovir, SFV+ MSCs would fuse with each other and collapse; Right panel, all the 8 monkeys were tested positive for SFV by PCR. (B) Tenfovir interferes with lentivirus infection. Left panel, SFV-negative culture showed a high infection rate at MOI 10 without Tenofovir treatment; Middle panel, with the addition of Tenofovir, the infection rate was very low; Right panel, without Tenofovir, infection rate could reach >80% at MOI 5 or 10. (C) Temporal removal of Tenofovir for 6 days beforehand allowed sufficient infection at MOI 10 and yet caused no cell fusion. Left panel, removal of Tenofovir for 2 days before infection; Middle panel, removal for 6 days did not cause cell fusion; Right panel, infection rate with various days of Tenofovir removal. Infection efficiency was all evaluated 3 days after infection. Original magnification 200×; $^{**}P<0.01$.



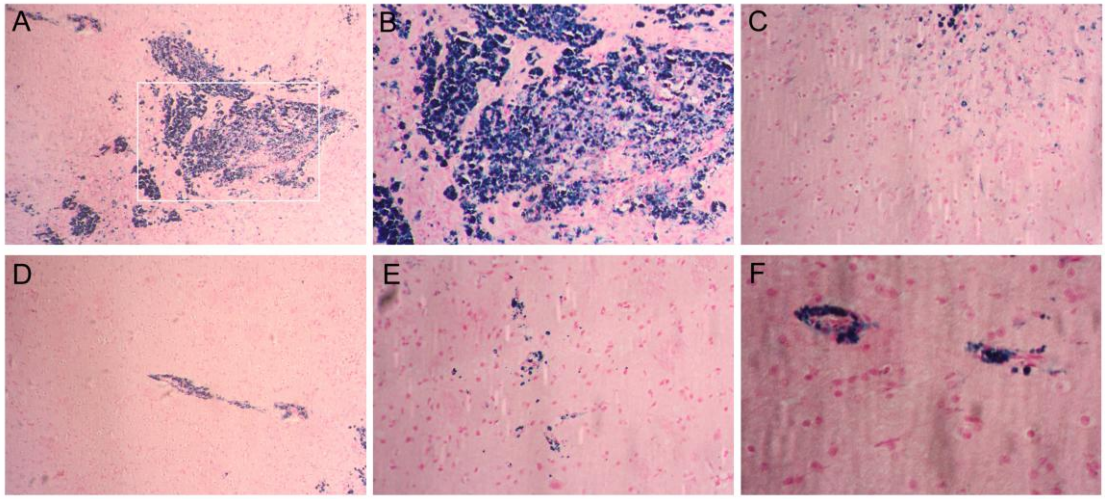
Supplementary Figure 4. Biological properties of ex vivo modified MSCs.

(A) The capability of MSCs to differentiate to adipocytes did not change after lentivirus infection. Red, Oil Red O staining to identify adipocytes. (B) Oil Red O was extracted using 100% isopropanol and quantified at 510 nm using a spectrophotometer. (C) The proliferation was slightly reduced by lentivirus infection as indicated by BrdU incorporation rate. (D) Labeling with Feridex slightly decreased GDNF production. (E) MSCs still showed a normal karyotype after lentivirus infection and Feridex labeling. (A), 200×; **P*<0.05.



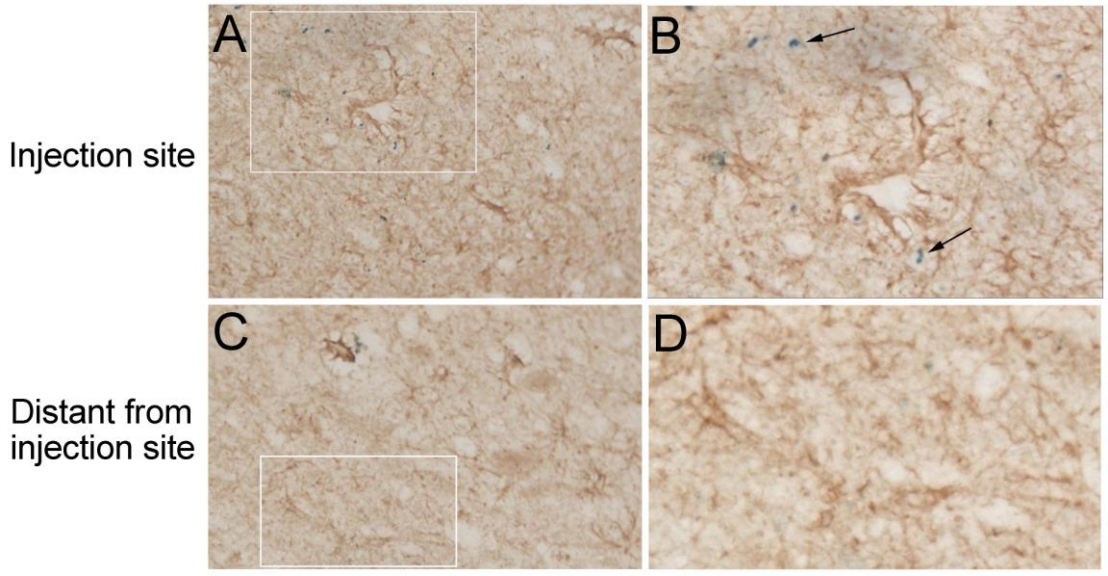
Supplementary Figure 5. GDNF levels in striatum and substantia nigra.

GDNF levels in brain tissues were determined by ELISA. (A) GDNF levels in the right striatum were higher than those in the left striatum of the 4 monkeys receiving unilateral GDNF-MSCs. (B) No significant difference was detected in substantia nigra in the left versus right side. *P<0.05 and **P<0.01.

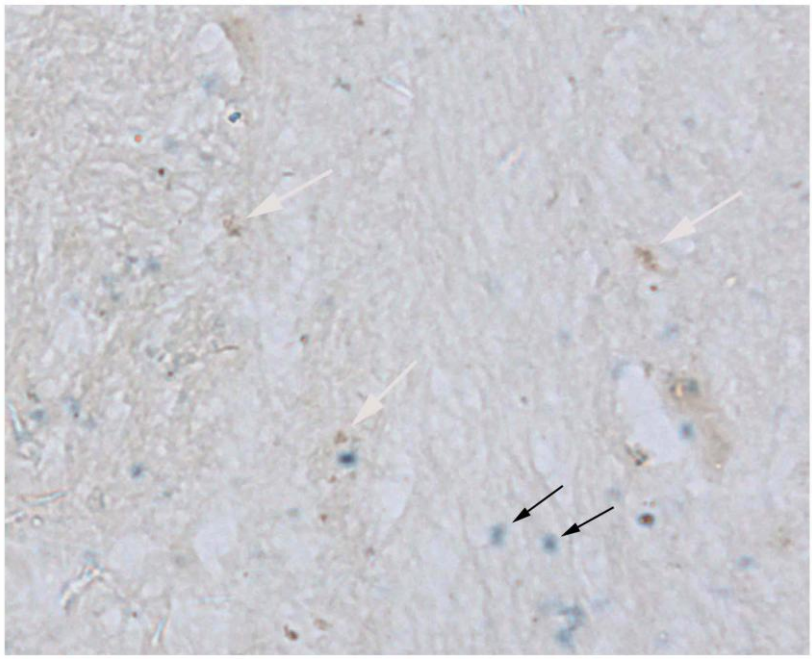


Supplementary Figure 6. Tracing of transplanted cells by Prussian blue staining.

The brain sections were stained by Prussian blue to trace the injected cells. (A and B) cell bolus deposited in striatum at different magnifications. (C) Some cells migrated out from the bolus. A small number of cells were seen at further distance on the ipsilateral side (D) and contralateral side (E and F), possibly by migrating through the vasculature systems. (A) and (D), 100×; (B-C) and (E), 200×; (F), 400×.

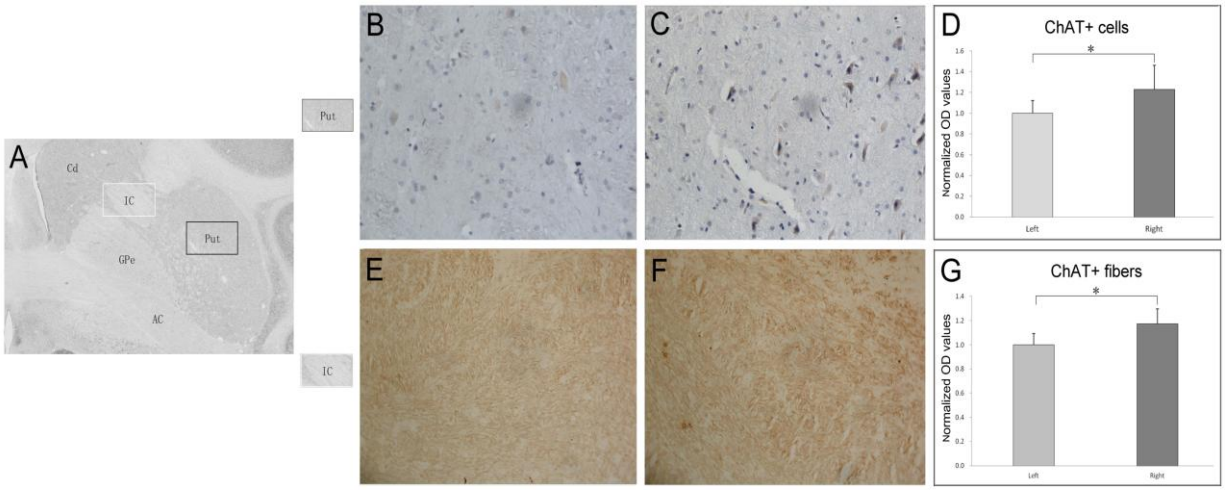


Supplementary Figure 7. Examination of astrocyte activation by GFAP staining. The brain sections were stained for GFAP by DAB method, following Prussian blue staining to reveal the transplanted cells. (A and B), transplantation site in the right striatum. (C and D), areas in the right striatum distant from the transplantation site. (B) and (D) are the magnified views of insets in (A) and (C), respectively. (A) and (C), 200×; (B) and (D), 400×. Arrows indicate the transplanted cells showing Prussian blue signals. Brown, GFAP; Blue, Prussian blue staining.



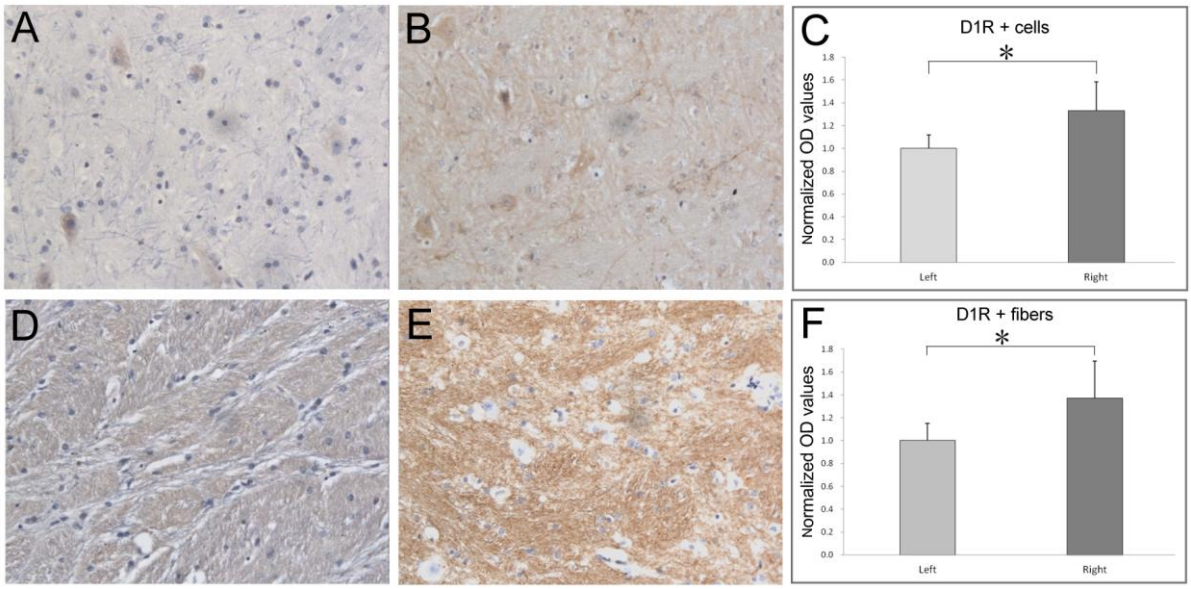
Supplementary Figure 8. Examination of microglia activation by Iba1 staining.

The brain sections were stained for Iba1 by DAB method, following Prussian blue staining to reveal the transplanted cells. White arrows, Iba1-positive cells; black arrows, transplanted cells showing Prussian blue signals. Magnification, 400×.



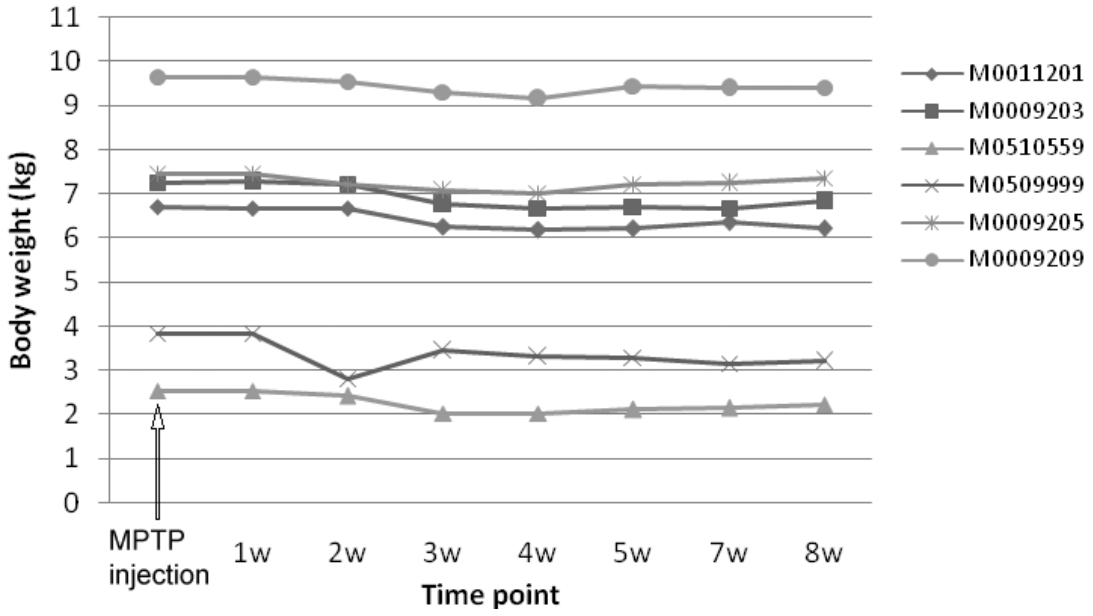
Supplementary Figure 9. Staining for choline acetyltransferase (ChAT).

The brain sections were stained for ChAT, followed by counterstaning of hematoxylin to reveal the cell nuclei. (A) The diagram of the striatum. (B and C) ChAT-positive cells in the left and right putman, respectively. The number of positive cells did not differ significantly between the left and right striatum. However, the staining intensities for the ChAT+ cells in the right striatum were significantly greater than those in the left stritum (D). (E and F) ChAT-positive fibers at the internal capsule in the left and right striatum, respectively. (G) The signal intensities on the fibers at internal capsule were higher in the right versus left hemisphere. (B and C), 400×; (E and F), 200 ×. Brown, ChAT+ signals; Blue, hematoxylin stained cell nuclei.



Supplementary Figure 10. Staining for dopamine receptor D1R.

The brain sections were stained for D1R, followed by counterstaning of hematoxylin to reveal the cell nuclei. (A and B) D1R-positive cells in the left and right putman, respectively. The number of positive cells did not differ significantly between the left and right striatum. However, the staining intensities for the D1R+ cells in the right striatum were significantly greater than those in the left striatum (C). (D and E) D1R-positive fibers at the internal capsule in the left and right striatum, respectively. (F) The signal intensities on the fibers at internal capsule were higher in the right versus left hemisphere. (A, B, D, and E), 400×; Brown, D1R+ signals; Blue, hematoxylin stained cell nuclei.



Supplementary Figure 11. The body weights of cynomolgus monkeys following MPTP treatment.

Supplementary Table 1. Quarantine report for the cynomolgus monkeys

Monkey ID	TB	SRV	SIV	SFV	Shigella	Helminths	Ectoparasites	Entamoeba histolyica	Salmonella
M0009205	–	–	–	+	–	–	–	–	–
M0009209	–	–	–	+	–	–	–	–	–
M0510559	–	–	–	+	–	–	–	–	–
M0509999	–	–	–	+	–	–	–	–	–
M0009203	–	–	–	+	–	–	–	–	–
M0011201	–	–	–	+	–	–	–	–	–

Note: "–": Negative; "+": Positive; TB, tubercle bacillus; RSV, respiratory syncytial virus; SIV, simian immunodeficiency virus; SFV, simian foamy virus.

Supplementary Table 2. Fluorescent antibodies used in flow cytometry

Fluorescent antibody	Clone NO.	Fluorescence	Source	Website
CD105	clone SN6	FITC	Serotec, Oxford,	http://www.serotec.com
CD73	AD2	PE	PharMingen, San Diego, CA,	http://www. pharmingen. com
STRO-1	--	PE	santa cruz, Biotechnology Inc.	http://www.scbt.com
CD106	51-10C9	FITC	PharMingen, San Diego, CA,	
CD90	5E10	FITC	PharMingen, San Diego, CA,	
CD14	MφP9	Percp	PharMingen, San Diego, CA,	
CD34	8G12	APC	PharMingen, San Diego, CA,	http://www. pharmingen. com
CD45	2D1	Percp	PharMingen, San Diego, CA,	
CD19	SJ25C1	FITC	PharMingen, San Diego, CA,	
HLA-DR	L243 (G46-6)	FITC	PharMingen, San Diego, CA,	

Supplementary Table 3. The transplant sites for cynomolgus monkeys

Monkey I.D. / Site/Volume	Three-dimension	M0011201	M0009203	M0510559	M0509999	M0009205	M0009209
Cd 1 (10μl)	AP	13.50	19.50	0.00	5.50	21.00	5.00
	Lat	4.39	4.96	4.73	5.57	5.17	4.90
	V	8.69	16.36	13.50	14.33	13.99	14.69
Cd2 (10μl)	AP	17.50	25.00	4.00	10.50	27.00	9.50
	Lat	5.37	4.88	4.45	5.01	4.34	5.03
	V	8.48	15.89	12.94	13.77	14.13	14.13
Cd3 (5μl)	AP	24.50	28.00	7.00	14.50	29.50	12.5
	Lat	5.30	4.88	4.45	5.01	4.34	5.31
	V	13.48	16.45	12.8	13.91	14.27	13.85
Put 1 (10μl)	AP	13.50	19.50	0.00	5.50	21.00	5.00
	Lat	5.48	10.36	10.85	12.10	12.17	11.33
	V	10.45	16.52	13.63	16.42	16.50	14.27
Put 2(10μl)	AP	17.50	24.50	4.00	10.50	27.00	9.50
	Lat	8.45	10.87	9.88	10.43	9.23	9.79
	V	11.08	17.56	15.72	15.17	16.64	16.36
Put 3 (10μl)	AP	22.50	27.50	7.00	14.50	29.50	12.50
	Lat	9.32	9.34	8.63	9.04	8.53	8.67
	V	17.35	20.35	16.00	17.67	16.5	17.34
SN (5μl)	AP	13.50	20.00	0.00	5.50	21.00	5.00
	Lat	4.16	3.34	3.34	3.20	3.36	3.78
	V	28.10	32.06	29.22	30.61	27.97	30.21

SN, Substantia nigra; Put, Putamen; Cd, Caudate nucleus;
AP, Antero-poster; Lat, Lateral; V, Ventral; (unit: mm)

Supplementary Table 4. Survival of transplanted cells

Monkey ID	Total No. of transplanted cells	No. of transplanted cells in Str	No. of surviving cells in Str	Percentage of surviving cells
M0009205	5.14×10^{6}	2.14×10^{6}	1.54×10^{5}	7.19%
M0009209	5.05×10^{6}	2.10×10^{6}	1.37×10^{5}	6.52%
M0510559	6.23×10^{6}	2.59×10^{6}	3.01×10^{5}	11.62%
M0509999	5.92×10^{6}	2.46×10^{6}	2.67×10^{5}	10.85%
M0009203	4.63×10^{6}	1.93×10^{6}	1.28×10^{5}	6.63%
M0011201	4.24×10^{6}	1.76×10^{6}	1.34×10^{5}	7.61%

Str, Striatum.

Supplementary Table 5. Comparison of the sensitivity to MPTP between different age groups

Monkey ID	Age(Y)	Weight (Kg)	The number of injections (n)	MPTP (mg/kg)	Total amount of MPTP (mg)	Scores
M0009205	8.5	7.46	21	2.1	15.67	11
M0009209	8.5	9.65	21	2.1	20.27	10
M0009203	8.5	7.25	21	2.1	15.25	12
M0011201	8.1	6.70	21	2.1	14.07	11
Mean / SD	8.4±0.2	7.77±1.3	21±0.0	2.1±0.0	16.31±2.72	11±0.82
M0510559	3.7	2.54	19	1.9	4.83	13
M0509999	3.8	3.84	16	1.6	6.14	14
Mean / SD	3.75±0.07	3.19±0.9	17.5±2.12	1.75±0.21	5.48±0.92	13.5±0.71

Note: The amount of MPTP was calculated according to the animal's body weight (0.1 mg/kg), and was administered at the concentration of 0.2 mg/ml through the lower limb vein for 16 to 21 times. The scores of each monkey were assessed by three trained staff independently. The animals were dosed until reaching a score over 10 on Primate Parkinsonian Rating Scale (PPRC). Compared to 8~10 age group, 3~4 age group required fewer times of injection and less amount of MPTP to achieve a score over 10. Moreover, the monkeys of 3~4 age group presented with more severe parkinsonian symptoms and higher PPRS scores. The results suggest that juvenile monkeys might be more sensitive to MPTP.

Supplementary Table 6. Biochemical survey of blood

Test items	Parameter values	Unit	Monkeys											
			Before MPTP injection						After MPTP injection					
			M00110201	M0009203	M0509999	M0510559	M0009205	M0009209	M00110201	M0009203	M0509999	M0510559	M0009205	M0009209
ALT	54.80±20.60	IU/L	52	48	29	35	42	38	38	27	19	50	72	47
TBIL	7.08±10.72	umol/L	3.4	3.3	4.3	2.8	3.6	2.5	2.9	3.8	2	1.6	2.3	3.1
TP	80.05±7 .81	g/L	71.2	73.65	68.42	73.74	69.68	78.16	69.42	70.29	72.39	66.75	72.21	74.99
ALB	48.13±7.38	g/L	36.9	34.82	39.62	32.68	34.33	42.72	37.87	39.43	42.54	38.46	40.49	39.95
G	46.47±14.38	g/L	31.2	31.46	30.5	31.4	30.16	33.21	29.89	30.86	29.85	28.29	31.72	35.04
A/G	0.98±0.13	---	1.21	1.24	1.26	1.28	1.37	1.41	1.15	1.28	1.43	1.36	1.28	1.14
ALP	687.10±263.1	IU/L	215	185	239	189	216	237	158	59	290	409	159	276
GGT	97.13±26.98	IU/L	127	147	118	98	148	68	94	106	81	98	88	38
AST	70.04±29.17	IU/L	49	65	46	64	71	63	46	38	43	64	54	47
CK	231.04±50.86	IU/L	257	267	132	157	237	146	167	294	77	1419	652	371
LDH	389.81±74.4	IU/L	178	248	214	178	183	167	231	208	846	720	159	186
CREA	87.17±20.57	umol/L	126	127	84	71	114	89	134	102	45	43	81	68
Urea	22.19±5.37	mmol/L	5.42	6.7	6.07	5.98	5.52	5.69	5.79	6.6	6.07	5.98	6.96	5.23
GLU	3.56±1.17	mmol/L	3.57	3.16	2.72	4.47	2.7	3.18	3.16	3.31	2.72	4.47	2.8	3.25
TG	0.81±0.31	mmol/L	0.38	0.29	0.15	0.37	0.27	0.29	0.31	0.36	0.15	0.37	0.28	0.32
TCH	3.17±0.44	mmol/L	1.78	2.74	3.14	3.16	2.82	2.57	2.18	1.94	3.14	3.16	2.73	1.52
K	4.62±1.49	mmol/L	3.67	4.38	4.26	3.67	5.58	4.87	4.1	3.8	4.26	3.67	4.42	6.6
Na	143.00±5.11	mmol/L	138	137	143	139	138	136	145	146	148	144	136	135
Ca	2.41±0.44	mmol/L	2.62	2.37	2.68	2.55	2.37	2.63	2.41	2.45	2.68	2.55	2.37	2.63
CL	107.64±2.91	mmol/L	101.7	93.6	110.4	91.7	95.6	94.8	103.8	102.7	101.4	96.2	94.8	96.2
P	2.05±0.49	mmol/L	1.22	1.24	1.17	2.08	1.09	1.11	1.7	1.5	1.7	2.08	0.94	1.22

Note: The blood biochemical parameters were normal before and after cell transplantation. Lactate dehydrogenase (LDH) and creatine kinase (CK) increased, and Creatinine (CREA) decreased in a few monkeys after MPTP treatment.

Supplementary Table 7. Biochemical survey of CSF

Test items	Parameter values	Unit	Monkeys (After MPTP injection)					
			M0011201	M0009203	M0510559	M0509999	M0009209	M0009205
sg-c	45-80	mg/dL	54	49	50	42	31	44
cl-c	118-128	mmol/L	146	116	129	124	121	125
pro	15-45	mg/dL	28	26	25	22	21	40

Note: All values were within the normal range after MPTP injection.

sg-c: Cerebrospinal fluid glucose; cl-c: Cerebrospinal fluid chlorine; pro: CSF protein.

Supplementary Methods

Cell senescence

The senescent cells were analyzed by histochemical staining for β-galactosidase activity, using Senescence Cells Histochemical Staining Kit (Sigma-Aldrich, Saint-Louis, Missouri, USA). Briefly, the cells were fixed and washed twice, followed by incubation for 24 h at 37℃ in freshly prepared staining solution. In the end, cells were washed with H2O, and observed by light microscopy. β-galactosidase activity was revealed by the presence of a blue, insoluble precipitate within the cells.

PCR assay of simian foamy viruses (SFV)

Genomic DNA was extracted from MSCs using a DNA mini kit (Qiagen Ltd, Crawley, UK) according to the manufacturer's instructions. PCR was performed on a PTC-100 thermal cycler (Bio-Rad Laboratories, Inc., Waltham, MA) using rTaq polymerase (Takara, Bio, Inc., Kyoto, Japan). The following primer pairs were used: round 1: forward 5'-AGGATGGTGGGGACCAGCTA-3'; reverse 5'-GCTGCCCC TTGGTCAGAGTG-3', and round 2: forward 5'-CAGTGAATTCCAGAATCTCTTC -3'; reverse 5'-CACTTATCCCACTAGATGGTTC-3' as previously described [1, 2]. Amplification was performed in an ABI 7700 Sequence Detection System (Applied Biosystems). Amplified products were separated by electrophoresis on a 1.5-2% agarose gel. Picture was acquired by Gel imaging system (Kodak).

Lentivirus infection of MSCs

Because of the presence of SFV in the MSCs, Tenfovir was added to maintain the proper culture. However, Tenofovir interfered with lentivirus infection and therefore was temporally removed from culture for 2-6 days before the infection process started. Seventy-two hrs after infection with lentivirus encoding GFP, the proportion of GFP-positive cells was determined by flow cytometry. Briefly, the cells were collected and washed, and re-suspended at 4℃ in PBS, and analyzed by flow cytometry. The SFV(-) MSCs were obtained from a fetal monkey and used as a control for the infection test.

Characterization of ex vivo modified MSCs

In order to determine the influence of lentivirus infection on the differentiation potential of MSCs, the cells were differentiated into adipocytes, and stained with Oil Red O. Oil Red O was extracted from the differentiated adipocytes using 100% isopropanol, and quantified by 510 nm absorbance using a spectrophotometer (Bio-Rad). After lentivirus infection, the proliferation potential of MSCs was analyzed by BrdU incorporation. Brief, 1×10^4 cells were seeded into each well of a 24-well plate and incubated for 24 hrs, and 10 μM BrdU (Sigma– Aldrich) was added in the last 12 hours. After medium change to remove BrdU, the cells were cultured for another 12 hrs and subsequently fixed and treated with 2 N HCl for 40 mins. Mouse anti-BrdU (Roche) and goat anti-mouse Texas Red (Jackson) were used for staining. Data were expressed as the percentage of BrdU-positive cells among the total cells in the 100x fields. Values were averaged from three independent experiments. After MSCs were labeled with Feridex, the levels of GDNF secretion were determined by using ELISA. Karyotype analysis also was re-tested after lentivirus infection and Feridex labeling.

GDNF levels in striatum and substantia nigra by ELISA

GDNF levels in striatum and substantia nigra were analyzed by ELISA. Tissues of bilateral striatum and substantia nigra were dissected out from a 2-mm thick coronal section of animal brain, and weighed. Tissues were homogenized in 1 ml extraction buffer (containing 10 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100) using a tissue-tearor, sonicated (ultrasonic for 3 seconds, interval 3 seconds, 20 cycles), and centrifuged at 14,000 ×g for 15 minutes at 4℃. The supernatant was stored at -80℃ until analyzed. The levels of GDNF were determined by ELISA according to the supplier's recommendations.

Body weight of cynomolgus monkeys

In this study, the body weight of all animals was monitored throughout the whole

experiments by electronic balance. Animals were placed in a transfer cage and weighed twice a week at fixed times.

Biochemical tests of blood and CSF

The samples of blood and CSF obtained from individual animals were collected. The serum was separated from blood cells by centrifugation and stored in -80 degrees until use. Centrifugation was required when cerebrospinal fluid was found to contain a small amount of blood. Blood biochemical test was performed by using HITACHI 7170 automatic biochemical analyzer (Hitachi Co., Ltd., Tokyo, Japan). Blood biochemical parameters included ALT, TBIL, BUN, CREA, Urea, TP, sodium, potassium, chloride etc., which reflected the functions of liver and kidney and homeostasis of body. Glucose, chlorine and protein in CSF were analyzed in CSF biochemical test.

Supplementary References

1. **Jones-Engel L, Engel GA, Schillaci MA, Rompis A, Putra A, Suaryana KG*, et al.* Primate-to-human retroviral transmission in Asia. *Emerg Infect Dis* 2005, 11(7): 1028-1035.**

2. **Jones-Engel L, May CC, Engel GA, Steinkraus KA, Schillaci MA, Fuentes A*, et al.* Diverse contexts of zoonotic transmission of simian foamy viruses in Asia. *Emerg Infect Dis* 2008, 14(8): 1200-1208.**