Full Methods

Culture of undifferentiated hESCs. hESC lines H9 (WA-09, XX, passages 35–45), H1 (WA-01, XY, passages 30-40) and iPS cell lines 2C6[27] (XY, passages 20-30) and SeV6 (XY, passages 20-30; derived from MRC-5 using non-integrating 4 factor Sendai vector system[31]) were maintained on mouse embryonic fibroblasts (MEF, Global Stem, Rockville, MD) in 20% knockout serum replacement (KSR, Invitrogen, Carlsbad, CA)-containing hESC medium as described previously[27].

Neural Induction. For FP-based midbrain DA neuron induction, a modified version of the dual-SMAD inhibition[13] and FP induction[12] protocol was used based on timed exposure to LDN193189 (100nM, Stemgent, Cambridge, MA), SB431542 (10μM, Tocris, Ellisville, MI), SHH C25II (100ng/ml, R&D, Minneapolis, MN), Purmorphamine (2μM, Stemgent), FGF8 (100ng/ml, R&D) and CHIR99021 (CHIR; 3μM, Stemgent). Note: for the FP induction protocol we refer to "SHH" treatment as exposure of cells to a combination of SHH C25II 100ng/ml + Purmorphamine (2μM). Cells were plated (35-40 x 10^3 cells/cm^2) and cultured for 11 days on matrigel (BD, Franklin Lakes, NJ) in Knockout serum replacement medium (KSR) containing DMEM, 15% knockout serum replacement, 2 mM L-glutamine and 10 μM β-mercaptoethanol. KSR medium was gradually shifted to N2 medium starting on day 5 of differentiation as described previously[13]. On day 11, media was changed to Neurobasal/B27/L-Glut containing medium (NB/B27; Invitrogen) supplemented with CHIR (until day 13) and with BDNF (brain-derived neurotrophic factor, 20n/ml; R&D), ascorbic acid (AA; 0.2mM, Sigma, St Louis, MI), GDNF (glial cell line-derived neurotrophic factor, 20ng/ml; R&D), TGFβ3 (transforming growth factor type β3, 1ng/ml; R&D), dibutyryl cAMP (0.5mM; Sigma), and DAPT (10nM; Tocris,) for 9 days. On day 20, cells were dissociated using Accutase® (Innovative Cell Technology, San Diego, CA) and replated under high cell density conditions (300-400k cells/cm^2) on dishes pre-coated with polyornithine (PO; 15μg/ml)/ Laminin (1μg/ml)/ Fibronectin (2μg/ml) in differentiation medium (NB/B27 + BDNF, AA, GDNF, dbcAMP, TGFβ3 and DAPT) until the desired maturation stage for a given experiment.

For rosette-based DA neuron induction we followed our previously described protocols[2] but used dual-SMAD inhibition to accelerate the initial neural induction step. In brief, hESCs were induced towards neural fate by coculture with irradiated MS5 cells in KSR supplemented with SB431542 and Noggin (250ng/ml; R&D), from day 2-8 and SHH+FGF8 from day 6-11 of differentiation. After 11 days in KSR, neural rosettes were manually isolated and cultured (P1 stage) in N2 medium supplemented with SHH, FGF8, BDNF and AA as described previously[2]. After 5-7 days in P1 stage, rosettes were again harvested mechanically and triturated following incubation in Ca^2/Mg^2-free Hanks' balanced salt solution (HBSS) for 1 h and replated on PO/Laminin/Fibronectin coated plates. Patterning with SHH/FGF8 was continued for 7 days at P2 stage followed by final differentiation in the presence of BDNF, AA, GDNF,

TGFb3 and dbcAMP as described above until the desired maturation stage for a given experiment (typically 5-7 days for transplantation studies or 32 days for *in vitro* functional studies).

Gene expression analyses. Total RNA was extracted during differentiation at days: 0, 1, 3, 5, 7, 9, 11, 13 and 25 from each condition of control LSB, LSB/SHH/FGF8 and LSB/SHH/FGF8/CHIR using the RNeasy kit (Qiagen, Valencia, CA). For microarray analysis, total RNA was processed by the MSKCC Genomic core facility and hybridized on Illumina Human ref-12 bead arrays according to the specifications of the manufacturer. Comparisons were performed among each days and conditions using the LIMMA package from Bioconductor (http://www.bioconductor.org). Genes found to have an adjusted P-value < 0.05 and a fold change greater than two were considered significant. Some of the descriptive microarray data analyses and presentation was performed using a commercially available software package (Partek Genomics Suite (version 6.10.0915)). For qRT-PCR analyses, total RNA at day 25 of each condition was reverse transcribed (Quantitech, Qiagen) and amplified material was detected using commercially available Taqman gene expression assays (Applied Biosystems, Carlsbad, CA) with the data normalized to HPRT. Each data point represents 9 technical replicates from 3 independent biological samples. Raw data of all the microarray studies are available at GEO http://www.ncbi.nlm.nih.gov/geo/ accession number: GSE32658.

Animal Surgery. All rodent and monkey procedures were performed following NIH guidelines, and were approved by the local Institutional Animal Care and Use Committee (IACUC), the Institutional Biosafety Committee (IBC) as well as the Embryonic Stem Cell Research Committee (ESCRO).

Mice. *NOD-SCID IL2Rgc* null mice (20–35 g; Jackson Laboratory, Bar Harbor, ME) were anesthetized with Ketamine (90mg/kg; Akorn, Decatur, IL) and Xylazine (4mg/kg Fort Dodge, IA). 6-hydroxydopamine (10µg 6-OHDA (Sigma-Aldrich) was injected stereotactically into the striatum at the following coordinates (in millimeters): AP, 0.5 (from bregma); ML, -2.0; DV, -3.0 (from dura). Mice with successful lesions (an average of > 6 rotations / minutes) were selected for transplantation. A total of 150 x 10^3 cells were injected in a volume of 1.5µl into the striatum at the following coordinates (in mm): AP, 0.5; ML, -1.8; DV, 3.2. The mice were sacrificed 18 weeks post transplantation.

Rats. Adult female Sprague-Dawley (Taconic, Hudson, NY) rats (180-230g) were anesthetized with Ketamine (90mg/kg) and xylazine (4mg/kg) during surgical procedures. Unilateral, medial forebrain bundle lesions of the nigro-striatal pathway were established by stereotaxic injection of 6-OHDA (3.6 mg/ml in 0.2% ascorbic acid and 0.9% saline, Sigma) at two sites[32]. Rats were selected for transplantation if amphetamine-induced rotation exceeded 6 rotations/min by 6-8 weeks post injection. 250 x 10^3 cells were transplanted into the striatum of each animal (Coordinates: AP +1.0mm, ML -2.5mm and V-4.7mm;

toothbar set at -2.5). Control rats received PBS instead. The surgical procedures were described previously[32]. Daily intraperitoneal injections of cyclosporine 15 mg/kg (Bedford Labs, Bedford, OH) were started 24 hours prior to cell grafting and continued until sacrifice, 20 weeks following cell grafting.

Primates. Two adult (17-18 yr old; 10-12 kg; female) rhesus monkeys were rendered hemiparkinsonian via carotid MPTP administration followed by weekly I.V. MPTP administration to create a bilateral parkinsonian syndrome[33]. Both animals displayed parkinsonian symptoms consistent with a moderately-severe lesion. On the day of transplantation surgery, animals were tranquilized with ketamine (3.0 mg/kg, IM) and dexdomitor (0.02-0.04 mg/kg IM), intubated to maintain a stable airway and anesthetized with isoflurane. They were then placed into a stereotaxic frame for surgery. Both rhesus monkeys underwent a single surgery with three intracranial injections of human FP-derived DA cultures based on stereotaxic coordinates[34]. Bilateral injections of cells (10ul/injection; 125,000 cell/ul) were performed at three sites (1-posterior caudate, 2-pre-commissural putamen and overlying white matter) for a total volume of 30µl per hemisphere. An infusion pump attached to a stereotaxic micromanipulator was utilized to deliver the cells at a rate of 1µl/min though a 50µl Hamilton syringe with 28 G needle. After the injections were completed, the needle was left in place for an additional 2-5 minutes to allow the infusate to diffuse off the needle tip before slowly retracting the syringe. Immediately following surgery, the animals received analgesics (buprenex, 0.01mg/kg IM, BID for 72 hours post surgery; meloxicam, 0.1 mg/kg SQ, SID for 72 hours post surgery) as well as an antibiotic (cephazolin, 25 mg/kg IM, BID) until 72-hours post-surgery. The animals received cyclosporine A (Neoral, Sandimmune) orally (30 mg/kg tapered to 15 mg/kg) once daily beginning 48-hrs prior to surgery until sacrifice, one month following transplantation.

Behavioral Assays. Amphetamine-induced rotations (mice and rats) and the stepping test (rat) were carried out before transplantation and 4, 8, 12 and 18 weeks after transplantation. Rotation behavior in mice was recorded 10 min after i.p. injection of d-amphetamine (10 mg/kg, Sigma) and recorded for 30 minutes. Rotation behavior in rats was recorded 40 min after i.p. injection of d-amphetamine (5 mg/kg) and automatically assessed by the TSE VideoMot2 system (Germany). The data were presented as the average number of rotations per minute. The stepping test was modified from [30, 35]. In brief, each rat was placed on a flat surface, its hind legs were lifted by gently holding up the tail to allow only the forepaws to touch the table. The experimenter pulled the rat backwards 1 meter at a steady pace. Adjusting step numbers from both contralateral and ipsilateral forepaws were counted. Data was presented as the percentage of contralateral / (contralateral + ipsilateral) adjusting steps. The cylinder test was performed by placing each animal in a glass cylinder and counting the number of ipsilateral versus contralateral paw touches (out of 20 touches) to the wall of the cylinder as described previously[3].

Tissue Processing.

Mice and Rats:

Animals (mice and rats) received overdoses of Pentobarbital intraperitoneally (50 mg/kg) to induce deep anesthesia and were perfused in 4% paraformaldehyde (PFA). Brains were extracted, post-fixed in 4% PFA then soaked in 30% sucrose solutions for 2-5 days. They were sectioned on a cryostat after embedding in O.C.T. (Sakura-Finetek, Torrance, CA).

Primates:

Animals were sacrificed under deep anesthesia with ketamine (10 mg/kg, IM) and pentobarbital (25 mg/kg, IV) via cardiac perfusion with heparinized 0.9% saline followed by fresh cold 4% PFA fixative (pH7.4). Immediately following primary fixation, brains were removed from the skull and post-fixed in 4% PFA, free-floating, for 24-36 hrs. They were then rinsed and re-suspended in 10% sucrose on a slow shaker at 4^{o}C, and allowed to "sink". The process was then repeated in 20% sucrose followed by 30% sucrose. Whole brains were cut coronally into 40um serial sections on a frozen sledge microtome and stored free-floating in cryopreservative medium at -20^{o}C.

Immunohistochemistry: Cells were fixed in 4% PFA and blocked with 1% BSA with 0.3% Triton. Brain tissue sections were washed in cold PBS and processed similarly. Primary antibodies were diluted in 1-5% BSA or Normal Goat Serum and incubated according to manufacturer recommendations. A comprehensive list of antibodies and sources is provided as **Suppl. Table 6**. Appropriate Alexa488, Alexa555 or Alexa647-conjugated secondary antibodies (Molecular Probes, Carlsbad, CA) were used with 4′,6-diamidino-2-phenylindole (DAPI) nuclear counterstain (Thermo Fisher, Rockford, IL). For some analyses biotinylated secondary antibodies were used followed by visualization via DAB chromogen .

HPLC Analysis. Reversed-phase HPLC with electrochemical detection for measuring levels of DA, HVA and DOPAC was performed as described previously[7, 36]. Culture samples were collected in perchloric acid at day 65 of differentiation. For some experiments DA was measured directly in the medium using the same detection system but following aluminum extraction of DA and its metabolites using a commercially available kit as described previously[36].

Electrophysiological recordings

Cultures were transferred to a recording chamber on an upright microscope equipped with a 40X water-immersion objective (Eclipse E600FN; Nikon); cultures were perfused with saline containing in mM: 125 NaCl, 2.5 KCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 2 CaCl, 1 $MgCl_2$, and 25 glucose (34 ^{o}C; saturated with 95%

0_2-5% CO_2; pH 7.4; 298 mOsm/L). The saline flow rate was 2-3 ml/min running through an in-line heater (SH-27B with TC-324B controller; Warner Instruments). Neurons were visualized by video microscopy with a cooled-CCD digital camera (CoolSNAP ES^2, Photometrics, Roper Scientific, Tucson, AZ). Cells selected for electrophysiological recordings had neuron-like shapes with fine branching neurites. Somatic whole-cell patch-clamp recordings in current clamp configuration were performed with a MultiClamp 700B amplifier (Molecular Devices). Signals were filtered at 1-4 kHz and digitized at 5-20kHz with a Digidata 1440A (Molecular Devices). Recording patch electrodes were fabricated from filamented borosilicate glass (Sutter Instruments) pulled on a Flaming-Brown puller (P-97, Sutter Instruments) and had resistances of 4-6 MΩ in the bath. Electrodes were filled with internal solution containing in mM: 135 K-$MeSO_4$, 5 KCl, 5 HEPES, 0.25 EGTA, 10 phosphocroeatine-di(tris), 2 ATP-Mg, and 0.5 GTP-Na (pH 7.3, osmolarity adjusted to 290-300 mOsm/L). The amplifier bridge circuit was adjusted to compensate for electrode resistance and monitored. Electrode capacitance was also compensated. If series resistance increased >20% during the recording, the data were discarded.

Cell Counts and Stereological Analyses. The percentages of marker positive cells at the FP (day 11), midbrain DA neuron precursor (day 25) and mature DA neuron stages (day 50 or later) were determined in samples derived from at least 3 independent experiments each. Images for quantification were selected in a uniform random manner and each image was scored first for the number of DAPI-positive nuclei, followed by counting the number of cells expressing the marker of interest. All data are presented as mean ± SEM. Quantification of human cells (identified with anti-hNA) and TH+ neurons within grafts was performed on every tenth section where a graft was identifiable. Cell counts and graft volume were determined using the optical fractionator probe and the Cavalieri estimator using the Stereo Investigator software (MBF bioscience, Vermont) as described previously[37]. Data are presented as estimated total cell number and total graft volume +/- standard error of means (SEM). Statistical analysis was performed using the Student t-test (comparing 2 groups) or ANOVA with Dunnett test (comparing multiple groups against control).

References to Supplementary Materials

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