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Supplemental Information

IAP-Based Cell Sorting Results in Homogeneous Transplantable Dopaminergic Precursor Cells Derived from Human Pluripotent Stem Cells

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Figure S1. Related to Figure 1 D

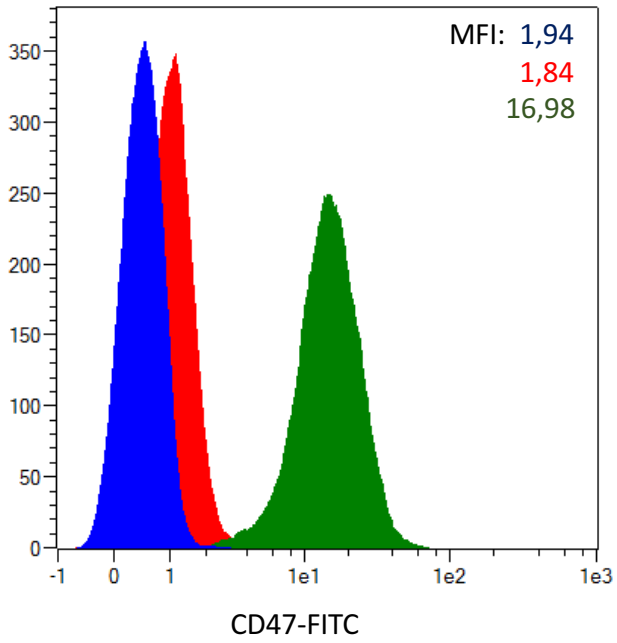

Figure S1. Related to Figure 1D. Blocking experiment to confirm specificity of REA220. Human PBMC were either stained with CD47-FITC (clone: B6H12, green) for 10min at 4°C or incubated with pure antibody clone REA220 at a final concentration of 25 µg/ml for 5 min at 4°C before adding CD47-FTIC (B6H12, red), unstained sample in blue. MFI: Mean Fluorescence intensity

Figure S2. Related to Figure 2 A/B

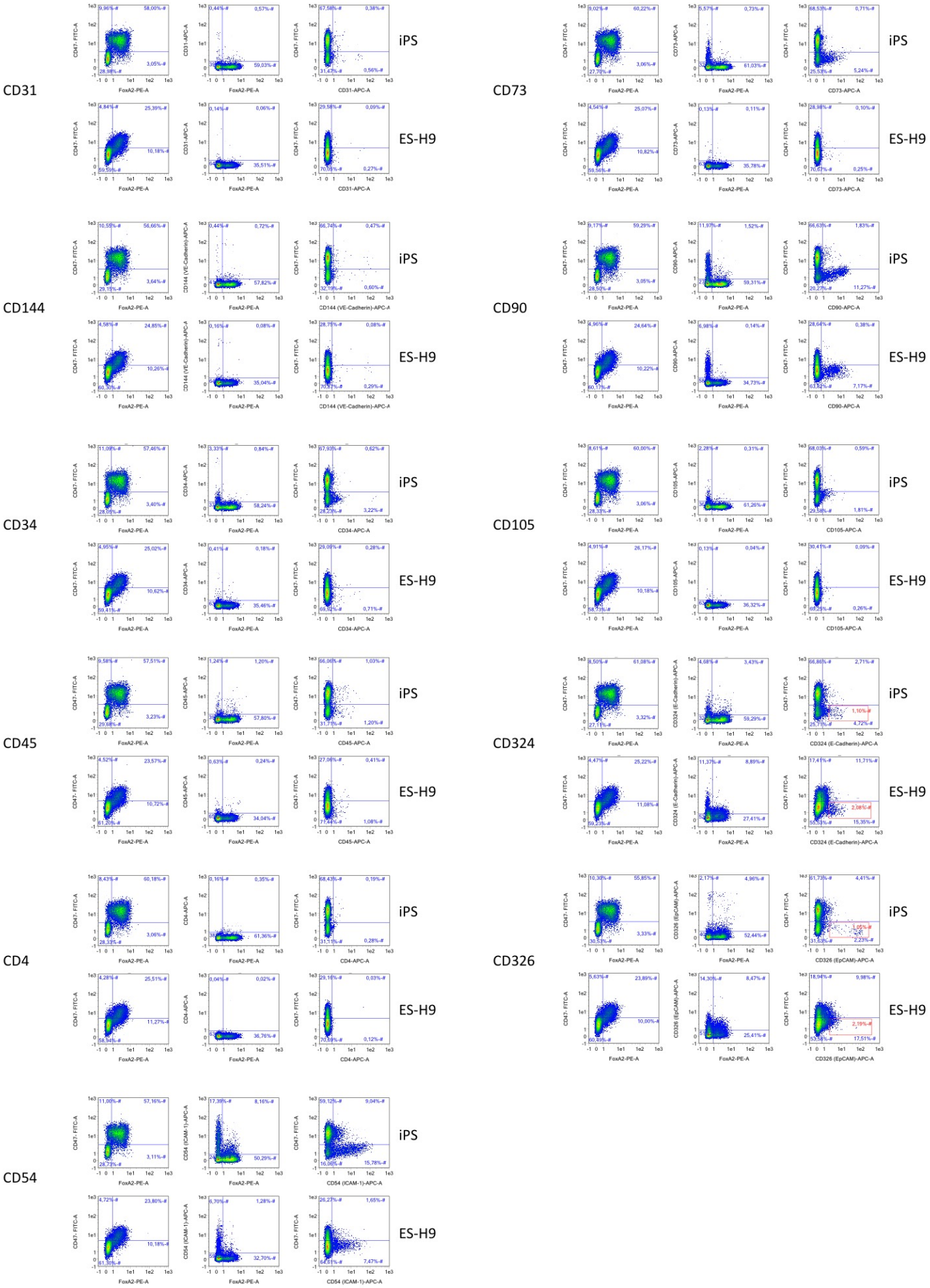

Figure S2. Related to Figure 2A/B. Expression of mesodermal and epithelial markers in relation to IAP+ or FoxA2+ target cells.

hESC (H9) and hiPSC (hFF-iPS) were differentiated towards mesDA progenitor cells and analyzed for marker expression on d16 of differentiation. Mesodermal (CD31, CD144, CD45, CD4, CD34, CD73, CD90, CD105) and epithelial (CD324, CD326, CD54) markers are depicted in relation to FoxA2 and IAP (CD47).

Figure S3. Related to Figure 3 E

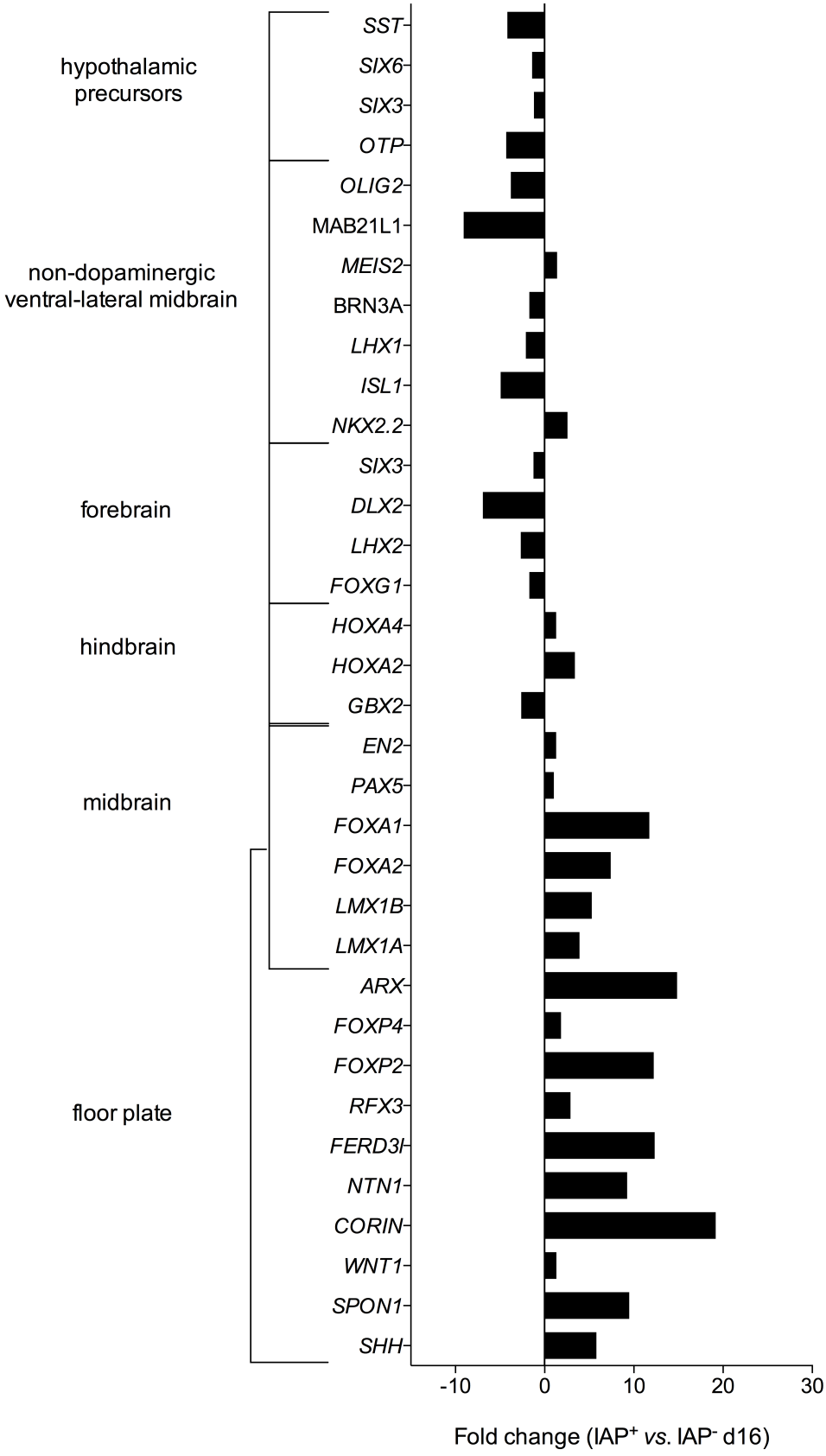

Figure S3. Related to Figure 3 E
Microarray analysis comparing IAP+ and IAP- cells sorted and analyzed at d16. Shown is the median fold change of expression of selected genes.

Figure S4. Related to Figure 4 B

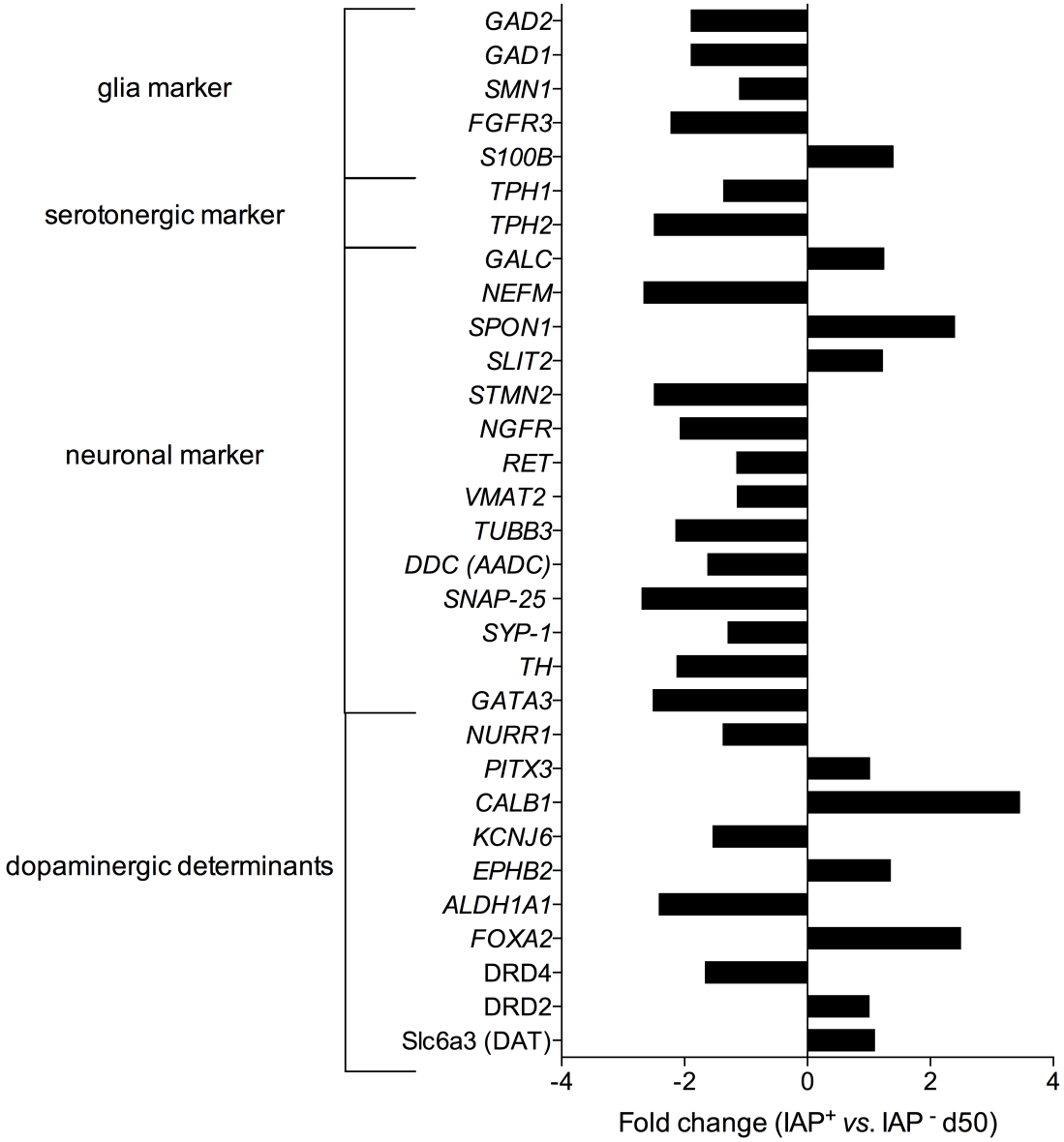

Figure S4. Related to Figure 4 B
Microarray analysis comparing IAP+ and IAP- cells sorted at d16, terminally differentiated and analyzed at d50. Shown is the median fold change of expression of selected genes.

Figure S5. Related to Figure 6

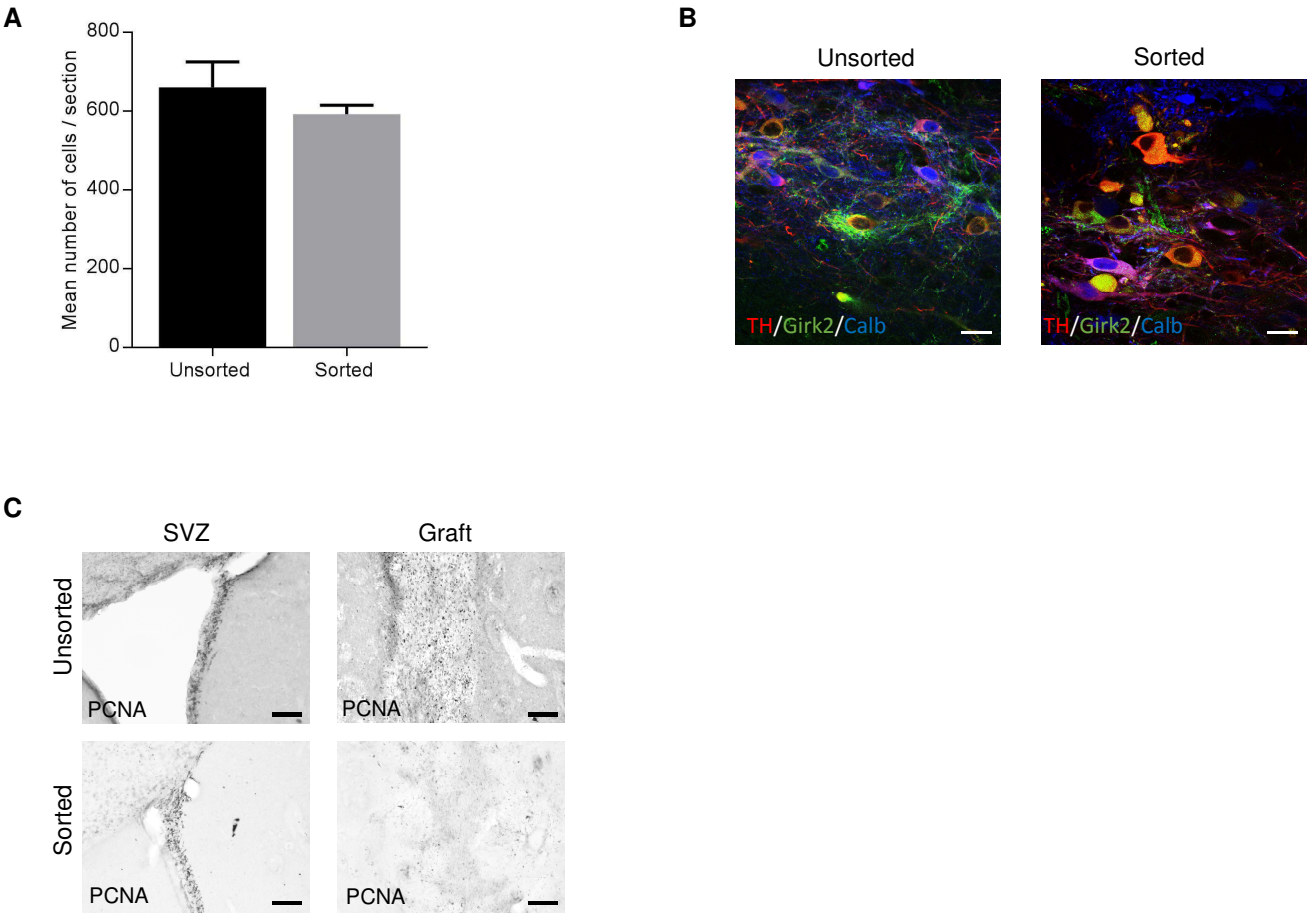

Figure S5. Related to Figure 6.
Human Nuclei (HuNu) counting in grafts from sorted and unsorted cells, unsorted n=3; sorted n=3; rats; mean ± SD (A) . B) The presence of neurons coexpressing TH and GIRK2 (red and green) and TH and Calbindin (red and blue) indicates that both midbrain DA neuron subtypes, A9 and A10, were contained in grafts of hESC-derived neurons, scale bars represent 20 µm. Staining of PCNA (C), scale bars represent 100 µm

Table S2

Antibody	Dilution	Species	Clone	Company
FOXA2	1:1,000	mouse	N17-280	BD Pharmigen
FOXA2	1:500	goat	polyclonal	Santa Cruz
LMX1a	1:2,000	rabbit	polyclonal	Millipore
TH	1:1,000	rabbit	polyclonal	Millipore
TUJ1	1:500	mouse / rabbit	TUJ1	Covance
hNCAM	1:100	mouse	ERIC 1	Santa Cruz
CD47	1:100	mouse	B6H12	BD Pharmigen
TH	1:1000	mouse	LNC1	chemicon MAB318
Calb	1:1000	rabbit	polyclonal	swant
Girk2	1:200	goat	polyclonal	abcam
PCNA	1:500	mouse	PC10	abcam ab29
5HT	1:10000	rabbit	polyclonal	Immunostar
goat anti mouse-Alexa Fluor 488	1:400	goat	polyclonal	Life technologies
goat anti mouse-Alexa Fluor594	1:400	goat	polyclonal	Life technologies
goat anti rabbit-Alexa Fluor 488	1:400	goat	polyclonal	Life technologies

Table S4

Symbol	Gene name	Primer Sequence
ActB	Beta-actin	CCTTGCACATGCCGGAG
		GCACAGAGCCTCGCCTT
Corin	Corin, serine peptidase	CATATCTCCATCGCCTCAGTTG
		GGCAGGAGTCCATGACTGT
EN1	Engrailed 1	CGTGGCTTACTCCCCATTTA
		TCTCGCTGTCTCTCCCTCTC
FoxA2	Forkhead box A2 (HNF3ß)	CCGTTCTCCATCAACAACCT
		GGGGTAGTGCATCACCTGTT
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	TTGAGGTCAATGAAGGGGTC
		GAAGGTGAAGGTCGGAGTCA
Lmx1a	LIM homeobox transcription factor 1a	CGCATCGTTTCTTCTCCTCT
		CAGACAGACTTGGGGCTCAC
Lmx1b	LIM homeobox transcription factor 1b	CTTAACCAGCCTCAGCGACT
		TCAGGAGGCGAAGTAGGAAC
SERT	Serotonin transporter (SLC6A4)	TGGACCCTCCATTCCACGTCCC
		GTCCTGGAGCCCCTTAGACCGG
SYP-1	Synaptophysin	ACCTCGGGACTCAACACCTCGG
		GAACCACAGGTTGCCGACCCAG
TH	Tyrosine hydroxylase	CGGGCTTCTCGGACCAGGTGTA
		CTCCTCGGCGGTGTACTCCACA
TPH2	Tryptophan hydroxylase 2	TCTGTGTCATGGCTTGGCTAAT
		TGAGTGGTTATCTGCCATTGGA

Table S5. Related to Figure 1

	iPSC d11	iPSC d16	ESC d11	ESC d16
IAP (CD47)	+	+	+	+
ITGA6 (CD49f)	+	+	+	+
BCAM (CD239)	+	+	+	+
MIC2 (CD99)	+	+	+	+
MRC (CD200)	+	+	+	+
B7H3 (CD276)	+	+	+	+
MCP (CD46)	+	+	+	+
DR5 (CD262)	+	+	+	+
ITGB1 (CD29)	+	+	+	+
NCAM (CD56)	+	+	+	+
ADAM10 (CD156c)	+	+	+	+
PTK7 (CCK-4)	+	+	+	+
AC133 (CD133/1)	-	+	-	-
GPIV (CD36)	-	+	-	-
HNK1 (CD57)	-	+	-	-
MDU1 (CD98)	-	+	-	-
ITGA2 (CD49b)	-	-	-	+
ITGAV (CD51)	-	-	-	+
293C3 (CD133/2)	+	+	-	-
PETA-3 (CD151)	-	-	+	+
CXCR4 (CD184)	-	-	+	+
BST2 (CD317)	-	-	+	+
ITGA5 (CD49e)	-	-	+	+
LAMP-3 (CD63)	-	+	-	+
BSG (CD147)	+	+	-	+
HSA (CD24)	+	+	-	+
PSANCAM	+	+	-	+
LFA-3 (CD58)	-	+	+	+
FAS (CD95)	-	+	+	+

Supplemental Experimental Procedures

Differentiation of hiPSC and hESC

The PSC were harvested with TrypLE™ for differentiation. Cells from feeder co-cultivation underwent a feeder removal step (Feeder Removal MicroBeads, Miltenyi Biotec). Single cells were seeded in low attachment plates ($2x10^6$ per 6well) to form EBs in DMEM/F12:MACS Neuro (1:1), 2 mM L-glutamine, N2 supplement (1:100; Gibco), NB-21 w/o vitamin A (1:50). ROCK-Inhibitor (Thiazovivin 2 µM, Miltenyi Biotec) was added for the first two days. On d4, the cells were plated on polyornithine (PO; 15 µg/ml; Sigma), Fibronectin (FN; 5 µg/ml; Biopur AG) and laminin (LN; 5 µg/ml; Sigma) coated plastic ware. From d0 to d9 SB431542 (10 µM), LDN193189 (100 nM), CHIR99021 (0.7-0.8 µM) (Stemgent or Miltenyi Biotec), hSHH-C24-II (200 ng/ml, Miltenyi Biotec) from d2-d9 Purmorphamine (0.5 µM, Miltenyi Biotec) was added to the medium. On d11 of differentiation, the cells were dissociated into single cells with Accumax (eBioscience) and either used for an experiment or replated on dry PO/LN/FN coated plates in droplets of 10,000 cells/µl in MACS Neuro, 2 mM L-glutamine, NB-21 w/o vitamin A (1:50) BDNF (20 ng/ml; Miltenyi Biotec), GDNF (10 ng/ml; Miltenyi Biotec) and L-ascorbic acid (200 µM, Sigma). Either cells were used for sorting on d16 or cultivated further. For the replating on d11, medium was supplemented with Rock-Inhhibitor (Thiazovivin 2 µM, Miltenyi Biotec). From d14 onwards, db-cAMP (0.5 µM; Sigma) or DAPT (2.5 µM, Miltenyi Biotec) was added to the medium for terminal differentiation.

Dorso-ventral patterning of neural progenitors: Ventralization of the cells was obtained by adding hSHH-C24-II to the medium. To drive floor plate patterning hSHH (200 ng/ml) and purmorphamine (0.5 µM from d2 onwards) were added. To obtain basal plate patterning only 100 ng/ml of SHH was added. For alar plate pattering no hSHH was added to the culture from d0 to d9. For roof plate fate, SB431542 and LDN193189 were removed from the culture at d4 to permit BMP and dorsalization.

Lesions and Transplantation Surgery

Surgical procedures were conducted under general anesthesia using a 20:1 fentanyl and medetomidine solution injected intraperitoneally (Apoteksbolaget, Sweden). The rats were made parkinsonian by injecting 6-OHDA at a freebase concentration of 3µg/ml into the medial forebrain bundle for complete lesion of the nigrostriatal dopaminergic pathway in the right hemisphere. At least 4 weeks post-lesion, cells were transplanted into the striatum using a microtransplantation approach as previously described (Rodter et al., 2000). For the 6 weeks survival study differentiated hiPSC, 2 × 1 µl deposits of 75,000 cells/µl (total of 150,000 cells) were transplanted to the striatum at the following coordinates relative to bregma: (1) A/P +0.5; M/L -3.0; D/V -4.0 and (2) D/V -5.0 (from dura); tooth bar -2.4. For the long-term study differentiated hESC, 2 x 2 µl deposits of 75,000 cells/µl (total 300,000 cells) at coordinates: (1) A/P +0.5; M/L +3.0; and (2) A/P +1.2; M/L -2.6 D/V -4.5 (from dura); tooth bar -2.4. Immunosuppressive treatment was given for the duration of the experiment, in the form of daily intraperitoneal injections of ciclosporin (10 mg/kg), beginning 1 day prior to transplantation.

Amphetamine-induced rotation

Rotational response was assessed using an automated system (Omnitech electronics) following i.p. injection of amphetamine (2,5mg/kg; Apoteksbolaget). The animals were recorded for 90 minutes and the results were expressed as net turns per minute. Only full body turns were counted with rotations towards the ipsilateral side to the lesion given a positive value. Only animals with more than 5 turns per minute were considered successfully lesioned.

Dopamine cell counts

DAB-developed immunohistochemistry was used to estimate the number of TH+ neurons within the graft core of each animal. Each TH+ positive neuron presenting a neuronal morphology was considered. To avoid errors in recounting the same cell, the upper and lower focal plane (using a 20x objective on a brightfield microscope) was imaged and overlaid for every section containing the graft in a given series. The total number of TH+ neurons per animal was corrected for the series number.

Graft volume

To estimate the graft volume in each animal, DAB-developed immunohistochemistry for hNCAM (Fig.5) or HuNu (Fig.6) was used. Every section containing the graft in a given series was imaged and the area covered by the graft in each section was calculated using ImageJ. The total area was then corrected for the series number to obtain the graft volume for each animal.

Immunohistochemistry

Animals were transcardially perfused with saline followed by ice cold 4% paraformaldehyde. Brains were post fixed for two hours, transferred to 25% sucrose for 48 hours and sectioned in a freezing microtome at a thickness of 35 µm. Immunohistochemistry was performed in 0,1M KPBS solution containing 0,25% Triton-X-100 and 5% serum for the species specific to the secondary antibody. Sections were incubated with primary antibodies overnight at room temperature followed by secondary antibodies for 1 hour in the same solution as above. Detection of the primary-secondary antibody complex was achieved by DAB precipitation.

Immunofluorescence

Cells grown in culture plates or imaging plates (PerkinElmer) were washed with PBS and fixed with 4% paraformaldehyde for 10 min (Merck). Antibody staining were performed in PBS/0.1% Triton-X-100 (Sigma-Aldrich)/10% goat or donkey serum. Cells were blocked for 30 min and primary antibodies were incubated overnight. After washing twice with PBS, secondary staining was conducted for 45 min (Invitrogen), followed by DAPI (Sigma) or DRAQ5 (eBioscience) staining for 10 min. For a complete list of antibodies and dilutions used, see Table S2.

Microarray Analyses

The microarray data is provided at NCBI GEO with accession number GSE74991. Each dataset is based on 3 independent experiments. Cells were lysed in RLT-buffer (Qiagen) and RNA was isolated using the RNeasy® Mini Kit (Qiagen). RNA samples, were quality-checked via the Agilent 2100 Bioanalyzer platform (Agilent Technologies). For the linear T7-based amplification step, 50 ng of each RNA sample was used. For generation of Cy3-labeled RNA, samples were amplified and labeled according to the manufacturer's protocol (Agilent Low Input Quick Amp Labeling Kit). Dye-incorporation and yield of cRNA were measured with the ND-1000 Spectrophotometer (NanoDrop Technologies). In the subsequent step up to 600 ng Cy3-labeled fragmented cRNA were hybridized overnight (17h, 65°C) to Agilent Whole Human Genome Oligo Microarrays 8x60K v2. The microarrays were washed twice before the scanning procedure. Detection of hybridized Agilent Microarrays was accomplished using Agilent's Microarray Scanner System. The data was normalized using quantile normalization and transformed to a logarithmic scale (log2). Sample groups were compared using ANOVA and Tukey post hoc tests. The false discovery rate (FDR) was controlled by applying Benjamini & Hochberg multiple testing correction. Significant expression differences were considered for pairwise group comparisons for reporters with p-values ≤ 0.05, at least two-fold median difference, and detectability of the signal ($p <= 0.01$, Resolver error model (Weng et al., 2006), in the majority of samples per group with higher expression. R/bioconductor (R version 3.0.1 (2013-05-16)) and packages therein were used for calculations and graphics. Table S3 lists the top 100 differentially expressed genes from the Microarray analysis of IAP+ (d16) vs IAP- (d16) and IAP+(d16) vs IAP+ (d50).

Functional grouping analysis by annotation enrichment analysis of differentially expressed genes (compared to all genes covered on the array) was performed to reveal overrepresented associations with known pathways using a Miltenyi Biotec software platform and databases curated from Gene Ontology (GO) and NetPath (Kandasamy et al., 2010; Weng et al., 2006). Heatmap images were generated with all the genes involved in the detected pathway using Multi- Experiment Viewer Software (MeV v4.8, TM4 Microarray Software Suite).

The reference data set GSE25931 was analyzed using 'geo2r' (http://www.ncbi.nlm.nih.gov/geo/geo2r/) to obtain the log2-transformed values of the fold change difference between adult DAs relative to hESC. Only reporters covered on both array platforms were used in the trend analysis.

Quantitative RT-PCR

RNA was isolated from cell cultures using the RNeasy Micro kit (QIAGEN). Reverse transcription was performed with Maxima First Strand cDNA Synthesis Kit (Thermo Scientific), using 1 μg of RNA from each sample. The cDNA was analyzed by quantitative PCR with Sybr green mastermix (Roche) on a LightCycler 480 instrument using a 2-step protocol. All quantitative RT-PCR (qRT-PCR) samples were run in technical triplicates, and the average Ct-values were used for calculations. Data are represented using the ΔΔCt method. All fold changes are calculated as the average fold change based on 2 different housekeeping genes (β-actin and GAPDH). Error bars on graphs illustrate variations of independent experiments, and data are shown as mean ±SD. Primers are listed in Table S4.

Supplemental References

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