Reversal of Cellular Phenotypes in Neural Cells Derived from Huntington’s Disease Monkey-Induced Pluripotent Stem Cells

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Supplemental Figures

A

5'LTR Flap Ubi HTT-Exon1 WPRE 3'LTR

5'LTR Flap Ubi GFP WPRE 3'LTR

B

HD-14 Pluripotency

OCT4 SSEA4

100 μm 100 μm

TRA-1-60 Alkaline Phosphatase

100 μm 100 μm

C

WT-2 Pluripotency

OCT4 SSEA4

100 μm 100 μm

TRA-1-60 Alkaline Phosphatase

100 μm 100 μm
Figure S1. Generation of pluripotent stem cells from HD and WT monkeys, Related to Figure 1

(A) HD monkey cell lines are derived from HD monkeys carrying mHTT transgene. This schematic illustrates the lentiviral vector carrying exon 1 of the human HTT gene with expanded CAG repeats. Cells derived from HD monkeys also carry GFP. Both vectors are regulated by human poly-ubiquitin-C promoter. LTR, long terminal repeats; Flap, HIV-flap sequence; GFP, green florescent protein; HTT, human huntingtin exon 1; Ubi, ubiquitin promoter; WPRE, woodchuck post-transcriptional regulatory element (Yang et al., 2008).

(B) Reprogrammed HD-14 cells from HD monkey form colonies and express stem cell pluripotency markers OCT4, SSEA4, TRA-1-60, and alkaline phosphatase. G-banding analysis shows normal diploid karyotype. (42; XX)

(C) WT-2 from WT monkey ESCs form colonies and express stem cell pluripotency markers OCT4, SSEA4, TRA-1-60, and alkaline phosphatase. G-banding analysis shows normal diploid karyotype. (42; XY)
**Figure S2. Characterization of neural cells derived from HD and WT monkeys, Related to Figure 1**

(A) Monkey NPCs except for HD-14 (shown in text) stained positively for Nestin, MSI1, PAX6, and SOX2 (red). Nuclear staining using Hoechst (blue).

(B) Monkey neural cells stained positive for β-III Tubulin (red), MAP2 (red), and DCX (red). Nuclear staining using Hoechst (blue).

(C) RT-qPCR shows elevated expression for NPC markers: SOX2, NES (Nestin), MSI1, and PAX6. Expression values were normalized to GAPDH. All graphs are relative to expression values in WT-2 ESCs and plotted as delta-delta CT method. Results were from three biological replicates. RT-qPCR samples run in duplicate. Data are represented as mean +/- SEM.
Table S1. Summary of HD and WT monkey cell lines included in this study, Related to Experimental Procedures

| Cell Line | HD-2 | HD-3 | HD-14 | WT-2 | WT-14 | WT-28 |
|-----------|------|------|-------|------|-------|-------|
| Phenotype | HD   | HD   | HD    | WT   | WT    | WT    |
| Source    | ICM/ESC | Fibroblast | DPSC | ICM/ESC | Fibroblast | ICM/ESC |
| CAG size  | 29   | 72   | 27, 65 | NP   | NP    | NP    |
| Mutant HTT Expression | X | X | X | ND | ND | ND |
| ICC Characterization (Fig 1:S2) | X | X | X | X | X | X |
| RT-qPCR Characterization (Fig S2) | X | X | X | X | X | X |
| FACS Analysis (Fig 1) | X | X | X | X | X | X |
| HTT Western Blot | X | X | X | X | X | X |
| In Vitro Differentiation | X | X | X | X | X | X |
| In Vivo Differentiation | NP | NP | X | X | NP | NP |
| H₂O₂/TUNEL | X | X | X | X | X | X |
| Reference | Putkhao et al., 2012 | Chan et al., 2010 | Yang et al., 2008 |

DPSC: Dental pulp stromal cells; ICM: Inner cell mass; ESC: embryonic stem cells; ICC: Immunocytochemistry; NP: Not performed; ND: Not detected

Table S2. Summary of FACS analysis in NPCs, Related to Figure 1

| Cell Line | FACS Results (% Positive) |
|-----------|---------------------------|
|            | Nestin | SOX2 | PAX6 | MS11  |
| HD-14      | 99.3   | 99.1 | 91.1 | 99.8  |
| HD-3       | 98.6   | 98.2 | 86.1 | 97.6  |
| HD-2       | 99.6   | 99.5 | 94   | 98.6  |
| WT-2       | 99.4   | 99.2 | 93.6 | 99.9  |
| WT-14      | 99.8   | 99.8 | 91.1 | 99.8  |
| WT-28      | 99.3   | 99.8 | 99.0 | 99.5  |
Supplemental Experimental Procedure

Reprogramming HD NHP iPSCs and Culture

HD monkeys were generated as described by Yang et al. Briefly, exon 1 of the human \textit{HTT} gene with expanded CAG repeats was inserted into a lentiviral vector. An additional lentiviral vector carrying EGFP was created for co-infection. Both vectors were under the regulation of the human UBC promoter (Yang et al., 2008). HD dental pulp stromal cells and fibroblasts were harvested from the dental pulp and skin of HD monkeys as described previously (Chan et al., 2010; Snyder et al., 2011). Teeth germs/buds were recovered from a HD monkey euthanized soon after birth (Snyder et al., 2011). Harvested teeth germs/buds were then digested in 3 mg/ml collagenase type I and 4 mg/ml dispase (Invitrogen) for 1 hr at 37°C. Single cell suspension was filtered through a 70-μm cell strainer and then cultured in DPSC culture medium [a-MEM (Invitrogen) supplemented with 20% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA), 2 mM L-glutamine, and 1 x penicillin/streptomycin (P/S, Invitrogen)] at 37°C with 5% CO$_2$. Harvested DPSCs and fibroblasts were infected by retrovirus expressing rhesus macaque Oct4, Sox2, and Klf4. At approximately 2-3 weeks post-retroviral transfection, a primate ES cell-like colony was selected based on morphology and mechanically passaged onto mouse fetal fibroblast (MFF) feeder cells with primate ES media. Cell lines are further described in Table S1.

Cytogenetic Analysis/G-Banding Analysis

Cytogenetic analysis was performed by Cell Line Genetics, LLC (Madison, WI). A total of 20 metaphases were analyzed, and images were captured using the CytoVision® digital imaging system (Applied Imaging).
Derivation and Culture of NHP-NPCs

iPSCs were mechanically dissociated from MFF feeder and were cultured in low-attachment petri dishes supported by MFF-conditioned ES cell medium without bFGF (R&D). After 7 days ES cell medium was replaced with derivation medium [DMEM/F12 (with 1x N2 (Invitrogen), 4 ng/ml bFGF (R&D), 2 mM L-glutamine, and 1 x P/S (Invitrogen)]. During this period cells cultured without passage with medium refreshed every 2 days. After 7 days neurospheres were plated onto P/L-coated [1 µg/cm² laminin (Sigma) and 20 ug/mL Poly-L-ornithine (Sigma)] cell culture dishes and expanded in neural proliferation medium [Neurobasal medium (Life Technologies) supplemented with 1 x P/S (Invitrogen) and 1x B27 (Life Technologies), 2 mM L-glutamine, 20 ng/ml bFGF (R&D), and 10 ng/ml mLIF (Chemicon)]. After 7-10 days neural rosettes were manually picked and seeded onto a fresh P/L-coated cell culture dish.

In Vivo Neuronal Differentiation

For in vivo differentiation to the neuronal lineage, WT-2 NPCs and HD-14 NPCs were dissociated and suspended at 50,000 cells/µL in artificial cerebrospinal fluid solution. By stereotactic injection, cell suspension was infused into the right and left hemispheres of the striatum (Anterior-Posterior = +0.74, Medial-Lateral +/-1.7, dorsal/ventral = -3.8, relative to Bregma). Cell transplantation was performed in Fox River SCID® mice (CB17/lcr-, Charles River Laboratories).

Immunocytochemistry Antibodies

Fixed slides were incubated overnight at 4°C with primary antibodies, including Nestin (1:1000; Millipore), SOX2 (1:500; Stem Cell Technologies), MSI1 (1:500; Millipore), PAX6 (1:300; Covance, Atlanta, GA), GABA (1:300; Sigma), β-III Tubulin (1:300; Millipore), Tyrosine
Hydroxylase (TH; 1:100; Millipore), MAP2 (1:500, Millipore), NR2A (1:200, Millipore), GluR1 (1:300) and mGluR5 (1:500), and mEM48 (1:50). Secondary antibodies included Alexa Fluor 594 (1:1000; Life Technologies), Alexa Fluor 647 (1:500; Life Technologies), and Hoechst 33342 (5 mg/ml) for DNA staining. Samples were examined using a microscope (Olympus BX51) equipped with an epifluorescent device.

**Histological Analysis**

At 12.5, 15, and 16 weeks following transplantation, mice were perfused transcardially with PBS followed by 4% PFA prepared in PBS. Brains were dissected and postfixed in the 4% PFA overnight. Fixed brains were cryopreserved with 15% sucrose overnight and switched to 30% sucrose for 8 hr to reach equilibrium. The brains were then embedded in OCT compound and cryosectioned coronally to 30-μm thick slices. Free-floating brain slices were incubated in blocking buffer (5% serum and 2% BSA) for 30 min, and then incubated overnight at 4°C with primary antibodies, including DCX (1:500; AB18723, AbCam), NeuN (1:500; MAB377 Chemicon), GABA (1:300; A2052, Sigma), dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP32; 1:200; 11365, Santa Cruz), GFAP (1:500; Chemicon), and mEM48 (1:50). Brain slices were washed with PBST (PBS + 0.2% Triton X-100) 3 times (10 min/time). Secondary antibodies (Alexa Fluor 594, A-21205; or Alexa Cy5, 81-6716) (1:500; Molecular Probes) were applied for 1.5 hr at room temperature. Slices were washed with PBST 2 times (10 min/time), and then incubated with Hoechst for 20 min. Subsequently, slices were mounted onto glass slides. Images were acquired using a Zeiss LSM 510 NLO META confocal microscope (Oberkochen, Germany).
Western Blot

Proteins were extracted using RIPA buffer and quantified by Bio-Rad DC Protein Assay (Pierce). Western blot was performed as described (Yang et al., 2008). Proteins were transferred to a PVDF membrane and probed with primary antibodies mEM48 (1:50), 1C2 (1:500), and γ-Tubulin (1:1000).

Fluorescence-Activated Cell Sorting (FACS) Analysis

Cells were dissociated using 1x Accutase (Life Technologies) and fixed in 1x BD FACS Permeabilizing Solution (BD Biosciences). Cells were washed following permeabilization. Cells were incubated with primary antibody for 1 hr, followed by 3 wash steps (5 min/wash) in 0.5% BSA/PBS. Cells were incubated with fluorochrome-conjugated secondary antibody for 1 hr in Falcon 5-mL round-bottom polystyrene tubes (BD Biosciences) and quantified on a FACSCalibur flow cytometer (BD Biosciences). All samples were gated to assess only single cells as determined by forward scatter area vs. side scatter area. Background fluorescence was subtracted using unlabeled cells, and channel compensation was performed using fluorochrome-labeled compensation beads (BD Biosciences). A total of 100,000 events were recorded. Quantification was analyzed using FlowJo analysis software (TreeStar).

Cell Stress and TUNEL Assays

Hydrogen Peroxide (H₂O₂): NPCs were treated with 5 mM H₂O₂ in culture medium for 24 hr. Following incubation cells were harvested and stained to assess caspase 3/7 activity using Muse™ Caspase-3/7 Kit (Millipore) according to the manufacturer’s instructions. Cell counts were analyzed using Muse Cell Analyzer (Millipore).

TUNEL: Effects of H₂O₂ were further assessed by TUNEL analysis. Samples were prepared on P/L-coated coverslips. Following H₂O₂ treatment for 24 hr, samples were fixed in 4% PFA and
stained using an In Situ Cell Death Detection Kit (Roche) to quantify the percentage of TUNEL-positive cells. For 3 biological replicates, 6 images were taken randomly and quantified using CellSens software (Olympus).

**Cytotoxicity Assay**

Monkey neural cells were differentiated for 21 days and then treated with 10 μM memantine (Sigma) for 24 hr. Vybrant Cytotoxicity Assay Kit (Life Technologies) was used according to the manufacturer’s instructions to assess cytotoxicity.

**Real-Time Quantitative PCR**

Total RNA from cell samples was prepared using TRIzol® (Life Technologies) followed by reverse transcription using a RNA-to-cDNA kit (Applied Biosystems). RT-qPCR was performed using Gene Expression Master Mix (Applied Biosystems) and TaqMan® gene expression primers on CFX96 Real-Time Detection System (Bio-Rad). Primer sequences are listed in Supplemental Methods.

**RT-qPCR TaqMan® Primers**

| Gene Symbol | TaqMan® Primer Context Sequence |
|-------------|--------------------------------|
| GAPDH       | TCCAGGAGCGAGATCCCTCCAAAAT      |
| MSI1        | TTTGAGCAGTTTGGGAAAGGGACG       |
| NES         | CCACGTACAGGACCCTCCTGGAGGC      |
| PAX6        | ATGCAGAAACAGTCACACGGGAGTGA     |
| POU5F1 (OCT4)| CCCTGGGGGTTCTATTTGGGAAGGT     |
| SOX2        | GGCCTCGGCGGAAAAACCAAGACG       |

**RT-qPCR SYBR Primers**

| Gene Symbol | Forward Primer | Reverse Primer |
|-------------|----------------|----------------|
| HTT Exon 1  | GCGACCTGGAAAAAGGCTG | CTGCTGCTGCTGGAAGGACT |
| Ubiquitin C | CCACCTCTGCACCTGGTCCTG | CCAGTTGGGAATGCAACAACTTTA |