Supplementary information, Data S1 Materials and methods

Direct reprogramming and cell culture

Human foreskin fibroblasts (CRL-2097, ATCC) or adult human dermal fibroblasts (AHDF, Cell Applications) were cultured in MEF medium (Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 0.1 mM non-essential amino acids, and 2 mM Glutamax) and transduced with lentiviruses encoding OCT4 only, SOX2 only, or OCT4 and SOX2. Lentiviral supernatants were produced and harvested as described (Yu et al., 2007). Briefly, 293T cells (Invitrogen) were plated at 5×10^6 cells per 100-mm dish and incubated overnight in a 37°C incubator. Cells were transfected with 5 μg pSin-EF2-OCT4-Pur or pSin-EF2-SOX2-Pur (Addgene plasmid 16579 or 16577, respectively) along with packaging mix (3 μg psPAX2 and 2 μg pMD2.G) (Addgene plasmid 12260 and 12259) and FuGENE HD Transfection Reagent (Roche), according to the manufacturer's instructions. The cells were then cultured in a 32°C incubator, after which supernatants were collected at 48 and 72 hours post-transfection and filtered through a 0.45-μm filter (Millipore) before use. After two rounds of transduction on days 0 and 1, cells were harvested and re-plated onto diluted (1/45) Matrigel (growth factor-reduced, BD)-coated 6-well plates at a density of 10,000 cells/well for CRL-2097 and 33,000 cells/well for AHDF. The next day, the medium was changed to human reprogramming initiation medium (RepM-hIni: Advanced DMEM/F12 supplemented with 10% KSR, 5% ES-FBS, 2 mM Glutamax, 0.11 mM β-mercaptoethanol, 3 μM CHIR99021 and 0.5 μM A83-01 (Stemgent)) for 2 weeks to support cell growth, and then to human neural reprogramming medium (RepM-hNeural: Advanced DMEM/F12 and Neurobasal medium were mixed 1:1 and supplemented with 0.05% bovine serum albumin, 1x N2, 1x B27, 2 mM Glutamax, 0.11 mM β-mercaptoethanol, 3 μM CHIR99021 and 0.5 μM A83-01). After 28–35 days, typical colonies were isolated and plated onto Matrigel-coated plates. Chemical cocktail, containing small molecules, including 3 μM CHIR99021, 0.5 μM A83-01, 0.2 mM sodium butyrate, 2 μM LPA, 2 μM Rolipram, and 2 μM SP600125, was added to the medium before use.

Reprogramming with episomal vectors was done as described (Okita et al., 2011). Briefly, 4×10^5 fibroblasts were electroporated with up to 6 μg of the episomal vectors (pCXLE-hOCT3/4-shp53-F, pCXLE-hSK and pCXLE-EGFP) using the Microporator Human Dermal Fibroblast (NHDF) Nucleofector™ Kit (Lonza), according to the manufacturer's instructions. The cells were cultured in MEF medium for 1 week and re-plated onto Matrigel-coated 6-well plates at a density of 10,000 cells/well for CRL-2097 and 33,000 cells/well for AHDF. The culture medium was replaced the next day with RepM-hIni with small molecules (3 μM CHIR99021, 0.5 μM A83-01, 0.2 mM sodium butyrate, 2 μM LPA, 2 μM Rolipram, and 2 μM SP600125) for 1 week and with RepM-hNeural with small molecules (3 μM CHIR99021, 0.5 μM A83-01, 0.2 mM sodium butyrate, 2 μM LPA, 2 μM Rolipram, and 2 μM SP600125) for another 2–3 weeks. The iNSC-like colonies were observed around 4–5 weeks after electroporation and were picked for further expansion and evaluation.

The isolated colonies were cultured in RepM-hNeural continuously and used for further analysis. Neural stem cells differentiated from HuES9 as described (Li et al., 2011) and undifferentiated HuES9 human embryonic stem cells were used as controls in some experiments.

In vitro neuronal differentiation

For spontaneous neuronal differentiation, cultured cells were dissociated completely by Accutase (Innovative Cell Tech.) and plated on poly-ornithine- (Sigma), fibronectin- (BD) and laminin (Invitrogen)-coated plates in neuronal differentiation medium, which is the same as RepM-hNeural but without CHIR99021 and A83-01, for 2 weeks. Medium was then switched to N2 medium (Elkabetz et al., 2008) with 10 ng/ml BDNF (R&D) and 10 ng/ml GDNF (R&D) for an additional 2 weeks. FGF2 and EGF were added for 4 days before initiating differentiation. For dopaminergic neuronal differentiation,

the dissociated cells were cultured and differentiated as described (Li et al., 2011). For peripheral neuronal differentiation, the dissociated cells were cultured for 2 days in differentiation medium containing 20 ng/ml BMP4 (R&D), then in 0.1 μM compound-E (EMD), 10 ng/ml human LIF (Sigma) and 0.5 μM all-trans-retinoic acid (Sigma) for 5 days, and finally in differentiation medium without compound-E for 2–3 weeks. For oligodendrocyte differentiation, the cells were cultured in ODC medium (Advanced DMEM/F12 supplemented with 1 x N2, 2 mM Glutamax and 0.11 mM β-mercaptoethanol) containing 1 mM all-trans-retinoic acid for 5 days then supplemented with 100 ng/ml SHH and 1X B27 without vitamin A for 10 days. 20 ng/ml FGF2 was further treated for an additional 10 days and then transferred into new culture plate coated with Matrigel. After transfer, ODC medium containing 1X B27 without vitamin A, 20 ng/ml PDGF-AA, 100 ng/ml IGF, 1 mM cAMP and 10 ng/ml NT3 treated for 10 days and then finally DMEM/F12 supplemented with 1X N1, 60 ng/ml T3 and 1 mM cAMP for 21 days (Hu et al., 2009). Half of the medium was replaced at least once every 2 days during differentiation. All reagents were purchased from Invitrogen if not otherwise specified.

Immunocytochemistry

Samples were washed once with D-PBS (Invitrogen, without Ca^{2+} and Mg^{2+}) and fixed with a 4% formaldehyde solution (Electron Microscopy Sciences) with 0.15% picric acid (Sigma-Aldrich) in D-PBS for 15 min, and then washed three times with D-PBS. Blocking and permeablization were done with a 3% BSA (Jackson ImmunoResearch) and 0.3% Triton X-100 (Sigma-Aldrich) solution in D-PBS for 1 hour at room temperature. All primary antibodies were diluted in 1% BSA and incubated overnight at 4°C. Samples were washed with 0.1% BSA in D-PBS for 1 hour with several changes. Secondary antibodies conjugated with Alexa-555 or Alexa-488 (Invitrogen) were added to the samples for 1 hour at room temperature. All images were taken using a Zeiss AX10 microscope equipped with an Axiocam HRm camera, and processed with Axiovision software (Zeiss). Primary antibodies used were Pax6 (Covance, 1:500, rb), PLZF (EMD chemical, 1:100, m), OTX2 (Abcam, 1:300, rb), Tuj1 (Covance, 1:5000, m or rb), Dcx (Santa Cruz, 1:200, gt), NeuN (Millipore, 1:50, m), Map2 (Abcam, 1:5000, chk), GFAP (Dako, 1:1000, rb), GABA (Sigma, 1:3000, rb), vGlut1 (Synaptic Systems, 1:100, guinea pig), Peripherin (Santa Cruz, 1:100, gt), TH (Millipore, 1:1000, rb), O4 (R&D, 1:100, m), Olig2 (Millipore 1:200, rb), and Synapsin1 (Millipore, 1:2000, rb).

Flow cytometric analysis

After culturing for more than five passages after isolation, the hiNSCs were washed with D-PBS and dissociated with Accutase (Innovative Cell Tech.). For TRA-1-60 staining, samples were weekly collected at the time points indicated, and HuES9 was used as a positive control. After harvesting, the cells were washed twice with ice-cold FACS buffer (HBSS supplemented with 10 mM HEPES, 2% FBS, and 0.1% sodium azide (Sigma-Aldrich)). To remove undissociated cell clusters, cells were passed twice through strainers (BD). Cells were aliquoted and incubated individually with Alexa Fluor 647-conjugated TRA-1-60 (BD), phycoerytherin (PE)-conjugated CD15 (BD), PE-conjugated CD133, PE-conjugated PSA-NCAM and A2B5 (all from Miltenyl Biotec) antibodies for 30 min at 4°C (all concentrations as suggested by the manufacturer). Appropriate isotype control antibodies were also incubated in a separate cell aliquot. After twice washing with five volumes of FACS buffer, cells were fixed and resuspended in 4% formaldehyde solution (Electron Microscopy Sciences) in D-PBS. More than 20,000 cells were sampled using FACSCalibur and CellQuest software (BD). To analyze the data, FlowJo software (Tree Star) was used.

Chromatin immunoprecipitation

Epigenetic marker changes in various samples were analyzed using a Magna ChIP G kit (Millipore). Briefly, histones and DNA were cross-linked with 1% formaldehyde. Chromatin with a DNA fragment length of 200–500 bp was obtained by sonication using Bioruptor (Diagenode). The same amount of chromatin solution was incubated with anti-normal rabbit IgG, anti-trimethyl-histone H3Lys4 (Millipore),

or anti-trimethyl-histone H3Lys27 (Millipore), as well as with magnetic protein-G beads. After overnight incubation at 4 °C, DNA fragments were isolated and purified. DNA fragments obtained without antibody were used as input controls, whereas DNA fragments obtained with normal rabbit IgG were applied as negative controls. Purified DNA solutions were analyzed by quantitative PCR.

Reverse transcription and quantitative PCR

Total RNA was extracted from various samples with the RNeasy Mini Kit with QiaShredder (Qiagen). One μg of total RNA per sample was reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad) and the cDNA was diluted with 130 μl of water. 1/50 of the diluted cDNA and 1 μl of purified DNA from chromatin immunoprecipitation were used for quantitative PCR with iQ SYBR Green Supermix (Bio-Rad) on 7900HT fast real-time PCR system (Applied Biosystems). All qPCR reactions were done in triplicate, and the expression data were normalized to *GAPDH* expression and analyzed using DataAssist software (Applied Biosystems). All primer sequences are listed in Supplementary Table 1.

Microarray analysis

The GeneChip® Human Gene 1.0 ST Array (Affymetrix, CA) was used for microarray hybridizations to examine the global gene-expression profiles of CRL-2097, ONE and control hNSC cells. In brief, NuGEN Ovation Pico WTA V2 and NuGEN Encore Biotin Module were used for labeling. The Hybridization protocol may be found in the GeneChip Expression Analysis Technical Manual 701021 Rev.3. Finally, signals were scanned by GCS3000 and Data was processed using Affymerix Expression Console V1.1.

Episomal copy-number detection

The episomal copy-number detection was carried out as described (Okita et al., 2011). Briefly, the cells were harvested, and DNA was extracted and used for quantitative PCR analysis. The pCXLE-hOCT3/4-shp53-F plasmid was used to generate a standard curve to determine the correlation between copy number and threshold cycle (Ct) values for *EBNA-1*. Then, the copy number of *EBNA-1* in each iPSC sample was estimated from the observed Ct values.

Electrophysiological recordings

The reprogrammed ONE cells were dissociated and plated onto glass coverslips coated with poly-L-ornithine and laminin. The coverslips were placed in the recording chamber with a volume of about 200 μl. The recording chamber was mounted on the stage of an Olympus IX 71 inverted microscope. The whole-cell configuration of the patch-clamp technique was used for recording macroscopic currents at room temperature (22 ± 1 °C). Signals were amplified using an Axopatch200B amplifier (Axon Instruments), and filtered below 2 kHz via a Bessel low-pass filter. Data were sampled and analyzed using pClamp10.2 software (Axon Instruments for Molecular Devices) and a Dell computer Optiplex in conjunction with a DigiData interface (1322A; Axon Instruments). The patch pipettes were pulled from standard wall glass of 1.5 mm OD (Warner Instruments) with final tip resistance of 5–9 MΩ. For the recording of voltage-gated currents and action potentials, we used the following composition of the intracellular solution (in mM): 140 K-gluconate, 5 NaCl, 1 $MgCl_2$, 0.5 EGTA, 10 HEPES; pH adjusted by KOH to 7.3; osmolarity 290 mOsm. For the initiation of voltage-gated currents, we used potential steps from −60 to +30 mV, at 20 mV intervals (Δ20 mV). The duration of each step was 100 ms and was initiated after hyperpolarization to -90 mV for 300 ms to remove current inactivation. Solution changes were achieved rapidly, within 50–100 ms, by moving an array of constantly flowing pipettes relative to the cell with a micromanipulator. A control pipette containing bath solution was used to rapidly wash off applied drugs. NMDA was purchased from Ascent Scientific.

Intracellular Ca^{2+} measurements

After differentiation into neurons, cells were loaded for 1 hour at room temperature with Fura-2/AM (4 μM) in imaging buffer containing (in mM): NaCl, 132; KCl, 4.2; $CaCl_2$, 1; D-glucose, 5.5; HEPES/Tris, 10; glycine, 0.01; adjusted to pH 7.4. Fura-2 fluorescence was excited by a xenon light source with the wavelength alternating between 350 and 380 nm, and emission monitored at 510 nm. Images were collected using a CCD camera mounted on a Zeiss Axiovert 35 microscope and analyzed with SlideBook 5.0 software.

Transplantation and histology with neonatal mice

The hiNSCs labeled with lentivial-EGFP using pWPXL (Addgene #12257) and purified by FACSAria (BD) were used for transplantation as reported (Li et al., 2011). Briefly, 2–3 μl of Accutase-dissociated cells (5×10^4 cells per μl) were injected into the lateral ventricle of neonatal (P2-3) mice (n = 10). Thirty days after transplantation, animals were anesthetized and perfused with 4% paraformaldehyde. Brains were isolated and postfixed in 4% paraformaldehyde overnight. After cryoprotection in 30% sucrose solution, brains were frozen and cut on a cryostat (40 μm). Sections were incubated in blocking solution (3% BSA and 0.3% Triton X-100 in PBS). Subsequently, primary antibody (Tuj1, 1:2000, Covance; DCX, 1:500, Santa Cruz; MAP2, 1:1,000, Sigma; NeuN, 1:200, Millipore; GFAP, 1:1,500, DAKO) solution was applied to the sections overnight at room temperature. After three PBS washes, sections were incubated with Alexa Fluor 555-conjugated secondary antibody solution for 30 min at room temperature. Sections were then counterstained with Hoechst dye 33342, and were mounted. Mice were intraperitoneally injected with a single dose of BrdU (100 mg/kg) and sacrificed after 2 hours. Images were captured using a confocal microscope (Zeiss LSM510, Carl Zeiss).

Table S1. Primers used in this study.

Name	Sequence (5' to 3')
RT-GAPDH-F	TGCACCACCAACTGCTTAGC
RT-GAPDH-R	GGCATGGACTGTGGTCATGAG
RT-PAX6-F	GTCCATCTTTGCTTGGGAAA
RT-PAX6-R	TAGCCAGGTTGCGAAGAACT
RT-NES-F	CAGGAGAAACAGGGCCTACA
RT-NES-R	TGGGAGCAAAGATCCAAGAC
RT-SOX1-F	CAGTACAGCCCCATCTCCAAC
RT-SOX1-R	GCGGGCAAGTACATGCTGA
RT-T-F	TCAGCAAAGTCAAGCTCACCA
RT-T-R	CCCCAACTCTCACTATGTGGATT
RT-SOX17-F	GTGGACCGCACGGAATTTG
RT-SOX17-R	GGAGATTCACACCGGAGTCA
RT-OCT4 total-F	GAGGAGTCCCAGGACATCAA
RT-OCT4-total-R	AATAGAACCCCCAGGGTGAG
RT-OCT4-endo-F	AGTTTGTGCCAGGGTTTTTG
RT-OCT4-endo-R	ACTTCACCTTCCCTCCAACC
RT-OCT4-exo-F	TGTCTCCGTCACCACTCTGG
RT-OCT4-exo-R	ATGCATGCGGATCCTTCG
RT-NANOG-F	AACGTTCTGCTGGACTGAGC
RT-NANOG-R	ATGCTTCAAAGCAAGGCAAG
ChIP-PAX6-F	CCTTCACTTGACCGCTCAAG
ChIP-PAX6-R	ACCCACTAATCACTCCGCAACA
ChIP-SOX1-F	GCGAGGAGACAGCACACC
ChIP-SOX1-R	CCTGATGCACAAACCACTTG
ChIP-OCT4-F	GAGCAGAAGGATTGCTTTGG
ChIP-OCT4-R	AAAACCGGGAGACACAACTG

The primers can be distinguished by RT- or ChIP- for quantitative gene expression or chromatin immuneoprecipitation, respectively.

Supplemental References

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