Stem Cell Reports, Volume *7*

Supplemental Information

Expandable and Rapidly Differentiating Human Induced Neural Stem Cell Lines for Multiple Tissue Engineering Applications

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Supplemental Figures:

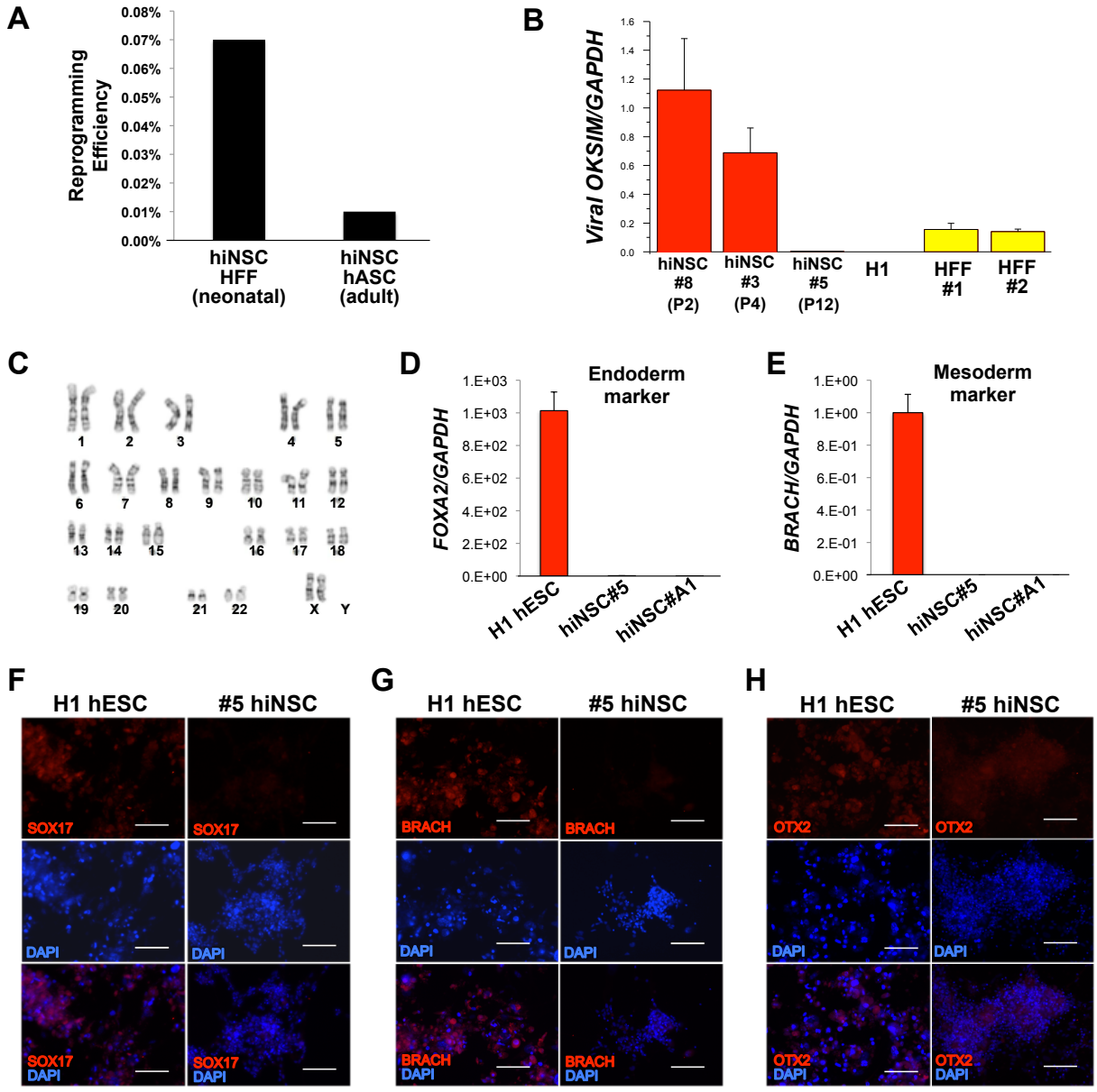

Figure S1. Related to Figure 2. Further characterization of hiNSC lines. (A) hiNSC reprogramming efficiency using various human cell types. (B) hiNSCs demonstrate a loss of exogenous transgene expression. Expression of the exogenous transgenes introduced via polycistronic lentiviral infection is lost upon increased passage of clonal hiNSC lines. Data represents means ± SD of 3 independent experiments. (C) hiNSCs display a normal karyotype. Example of a karyotype from resulting hiNSC line A1. (D,E) hiNSC colonies do not express markers of other germ layers. qRT-PCR analysis of H1 hESC compared to several hiNSC clonal lines demonstrates that hiNSCs do not express markers of endodermal (FOXA2) (D) or mesodermal (BRACHYURY) (E) lineages. Data represents means ± SD of 3 independent experiments. (F-H) hiNSCs cannot be induced to form endo- or mesodermal cell lineage. hESCs or hiNSCs were induced to specify endodermal, mesodermal or ectodermal lineage using a human pluripotent stem cell functional identification kit (R&D Systems). In response to specific germ layer inducing factors, hESCs differentiated into (F) SOX17-, (G) BRACHYURY- and (H) OTX2-positive cells, respectively, while hiNSCs did not express markers from endodermal nor mesodermal germ layers (scale bar, 100 uM).

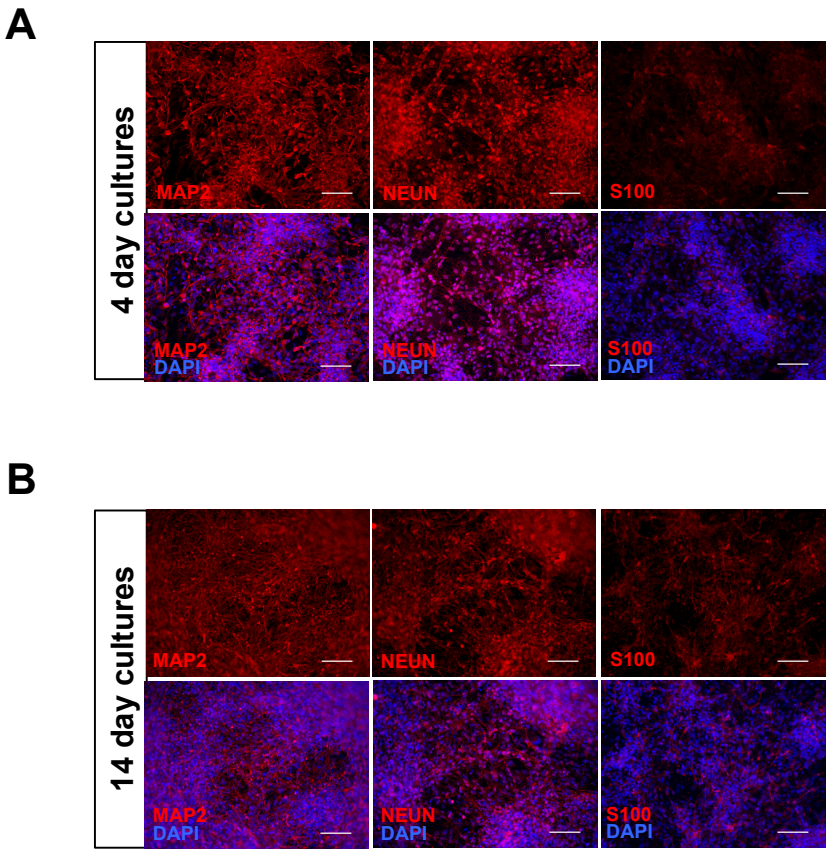

Figure S2. Related to Figure 3. Differentiated hiNSCs express multiple markers of neuron and glial differentiation. Spontaneously differentiated hiNSCs express other neuronal and glial markers including MAP2, NEUN and S100 after 4 and 14 days in culture (scale bar, 100 uM).

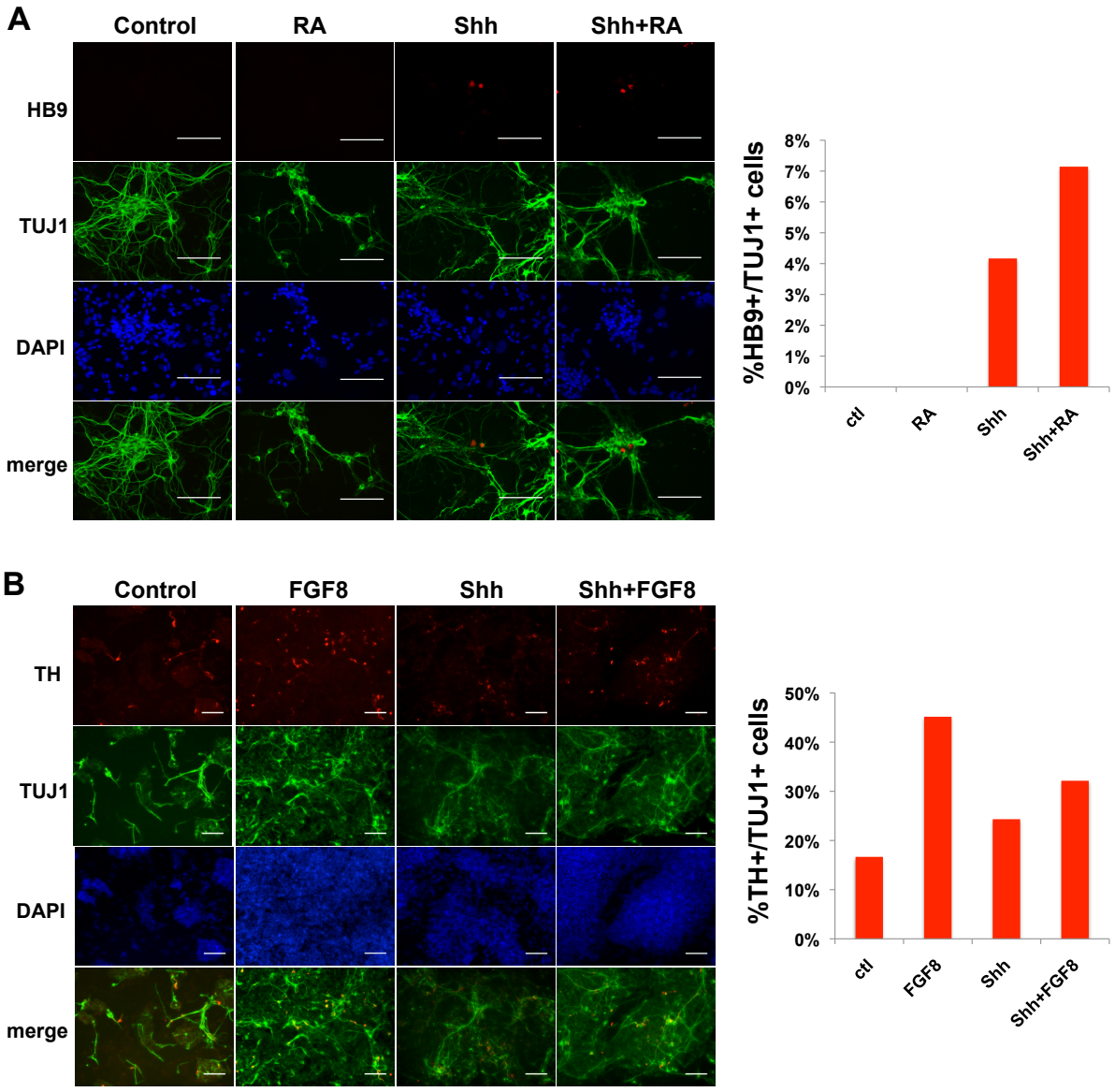

Figure S3. Related to Figure 3. hiNSCs can be directed to specific subtypes using growth factors: guidance of hiNSCs toward motor or dopaminergic neuron lineage. The addition of Shh and RA to Neurobasal media results in an increase in expression of HB9, a marker of motor neurons, after one week in culture (A). The addition of FGF8 and Shh to Neurobasal media results in an increase in expression of TH, a marker of dopaminergic neurons, after one week in culture (B).

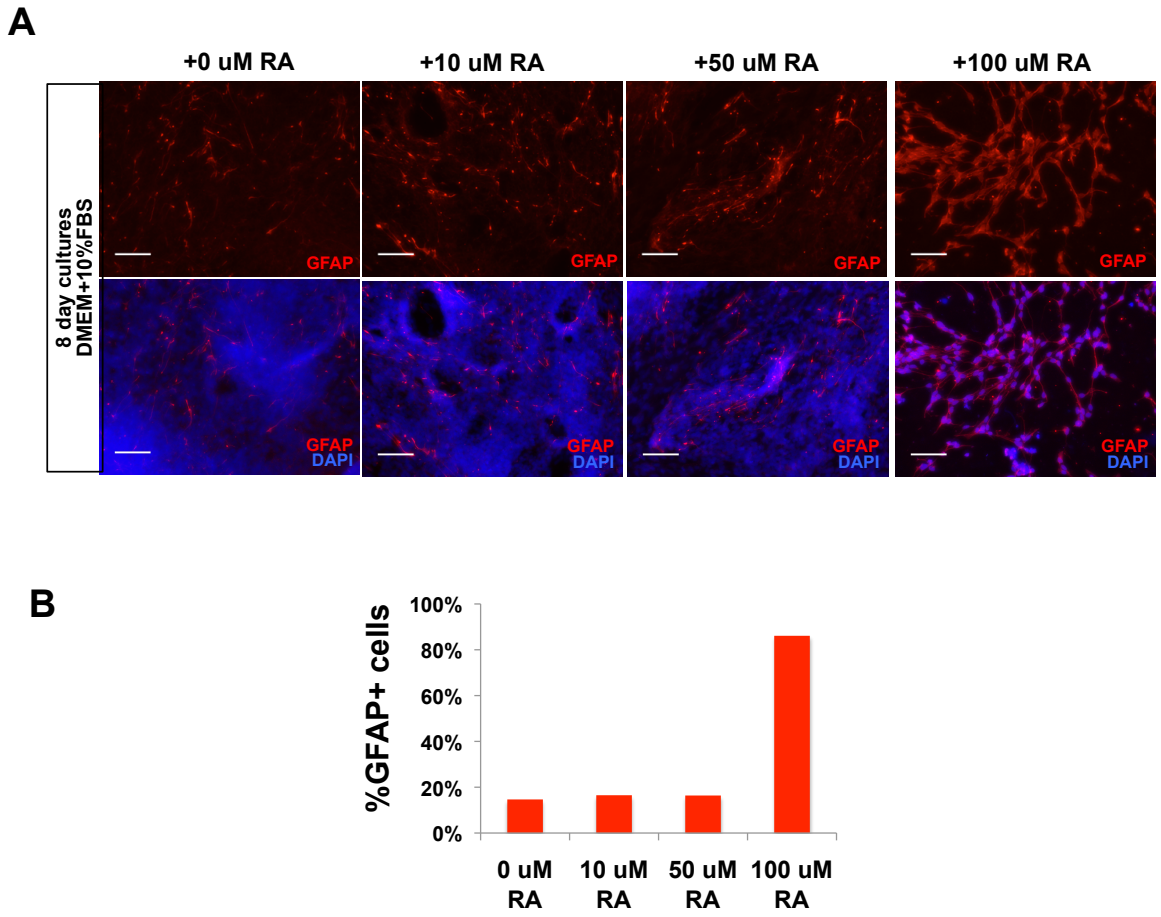

Figure S4. Related to Figure 3. hiNSCs can be directed to specific subtypes using growth factors: guidance of hiNSCs toward glial lineage. The addition of RA to DMEM+10%FBS media results in a dramatic increase in glial marker GFAP expression, after 8 days in culture.

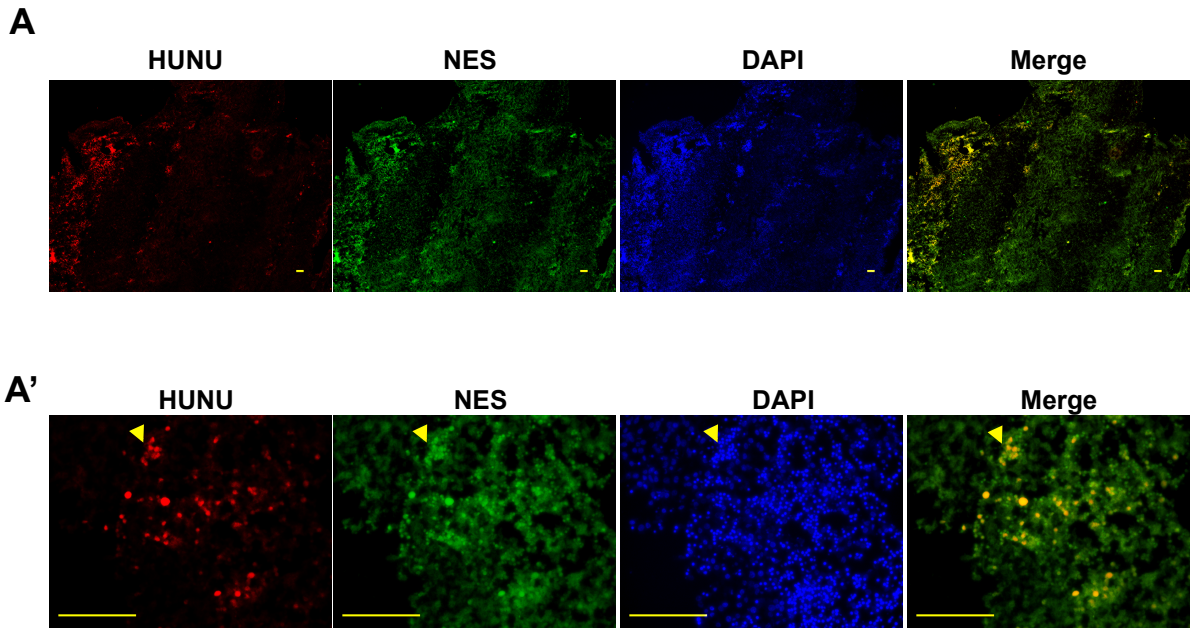

Figure S5. Related to Figure 5. Injected hiNSCs migrate out of the neural tube and engraft in multiple locations. hiNSCs were injected into early stage chick embryos, and allowed to grow for 6 days. Frontal sections through the spinal cord demonstrate the presence of human cells as indicated by human nuclear antigen (HUNU) immunostaining, which co-localizes exclusively with neural stem cell marker NESTIN (scale bar, 100 uM).

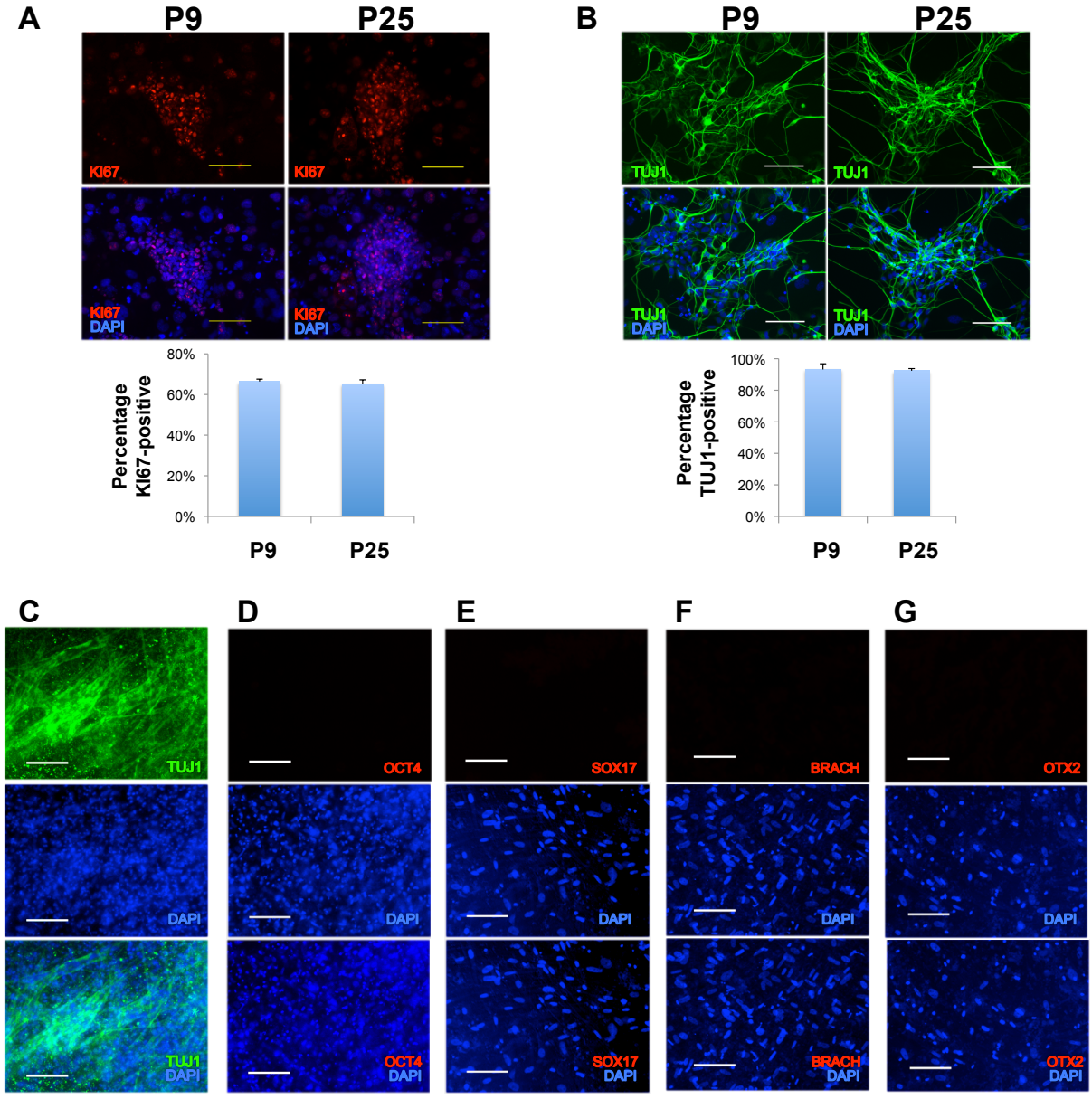

Figure S6. Related to Figures 2 and 3. hiNSCs demonstrate phenotypic stability. hiNSCs retain proliferative (A) and neurogenic differentiation capacity (B) upon being frozen, thawed and passaged multiple times. hiNSC lines were frozen as colonies at passage 3 (P3) and subsequently thawed and expanded on MEF feeder layers for multiple passages and analyzed for KI67 expression (A). To induce differentiation, hiNSC colonies were enzymatically dissociated and subcultured on gelatin substrate for 4 days and assayed for TUJ1 expression (B). Data (A,B) represents means ± SD of 3 independent experiments. hiNSCs in long term culture do not spontaneously induce expression of pluripotency marker OCT4 or germ layer markers. hiNSC lines were cultured *in vitro* on gelatin (in Knockout DMEM, 20% xeno-free serum replacement, 1% Glutamax, 1% antibiotic-antimycotic, and 0.1 mM β-mercaptoethanol, media which could allow for the growth of any potential pluripotent cells resident in this population) for 16 weeks then assayed for neuronal marker TUJ1 (A), pluripotent marker OCT4 (B), endodermal marker SOX17 (C), mesodermal marker BRACHYURY (D) and ectodermal marker OTX2 (E). Only neuronal marker expression was detectable after this extended period of time in culture (scale bar, 100 uM).

Supplemental Tables:

Host	Antigen	Vendor	Catalog #
Rabbit	OCT4	Stemgent	09-0023
Rabbit	SOX2	Stemgent	09-0024
Rabbit	NANOG	Stemgent	09-0020
Mouse	SSEA4	Stemgent	09-0006
Mouse	TRA-1-81	Stemgent	09-0011
Rabbit	PAX6	Stemgent	09-0075
Rabbit	SOX1	Abcam	ab87775
Rabbit	NESTIN	Sigma	N5413
(APC-conjugated)	CD133	Miltenyi Biotec	130-098-829
Rabbit	KI67	Abcam	ab15580
Rabbit	TUJ1	Abcam	ab18207
Mouse	TUJ1	Sigma	T8578
Mouse	GFAP	Sigma	G3893
Rabbit	GFAP	Sigma	G9269
Mouse	TH	Sigma	T1299
Rabbit	SLC32A1/VGAT	Sigma	SAB2700790
Rabbit	VGLUT2	Sigma	V2514
Rabbit	VGLUT1	Sigma	V0389
Mouse	GEPHYRIN	Abcam	ab124385
Mouse	PSD95	Sigma	p246
Rabbit	SYNAPTOPHYSIN	Abcam	ab32594
Rabbit	PAN NAV	Alomone	ASC-003
Rabbit	S100β	Millipore	04-1054
Rabbit	MBP	Millipore	AB980
Mouse	O4	Millipore	MAB345
Mouse	HUNU	Millipore	MAB1281
Cy3-conjugated mouse	HUNU	Millipore	MAB4383C3
Mouse	HB9	DSHB	81.5C10
Mouse	NF (sensory/motor)	DSHB	E1.9
Rabbit	MAP2	Sigma	M3696
Rabbit	NEUN	Abcam	ab104225
Rabbit	S100	Abcam	ab76729
Mouse	MHC	DSHB	MF20
Mouse	ISLET1/2	DSHB	39.4D5
Mouse	Schwann cell protein	DSHB	4E2(3G2)
Alexa 647 conjugated	Bungarotoxin	Invitrogen	B-13423
Goat (Alexa 488 conjugated)	Rabbit IgG	Invitrogen	A-11070
Goat (Alexa 594 conjugated)	Rabbit IgG	Invitrogen	A-11072
Goat (Alexa 488 conjugated)	Mouse IgG	Invitrogen	A-11017
Goat (Alexa 594 conjugated)	Mouse IgG	Invitrogen	A-11020

Table S1. Table of antibodies used for immunostaining.

Gene	Accession No.	Sequence 1 (5' → 3')	Sequence 2 (5' → 3')
endo *OCT4*	NM_002701.5/KF880691.1	AAACCCTGGCACAAACTCC	GACCAGTGTCCTTTCCTCTG
endo *SOX2*	NM_003106.3/JQ231229.1	CACATGTCCCAGCACTACC	CCATGCTGTTTCTTACTCTCCTC
NANOG	NM_024865.3	TCCTTGCAAATGTCTTCTGCT	CAGGGCTGTCCTGAATAAGC
PAX6	NM_000280.4	TCCGTTGGAACTGATGGAGT	GTTGGTATCCGGGGACTTC
SOX1	NM_005986.2	ATTATTTTGCCCGTTTTCCC	TCAAGGAAACACAATCGCTG
SOX11	NM_003108.3	TTTTCAAGCTCCCTGCAGTT	AGGGACCATTGCAACTTTTG
OLIG1	NM_138983.2	TGGTTACGCTACTTTTGGGG	CCAGTGTTTTGTCGCAGAGA
OLIG2	NM_005806.3	CTGGCGTCCGAGTCCAT	CCTGAGGCTTTTCGGAGC
MUSASHI	NM_002442.3	GTGAAGGAGTGTCTGGTGATG	GATTGCGCCAGCACTTTATC
DCX	NM_000555.3	TCAGGACCACAGGCAATAAA	AGACCGGGGTTGTCAAAAA
PLZF	NM_006006.4	TTCTCAGCCGCAAACTATCC	ATAACGAGGCTGTGGAGCAG
CD133	NM_006017.2	TTTTGGATTCATATGCCTTCTGT	ACCCATTGGCATTCTCTTTG
NESTIN	NM_006617.1	AGAACTCCCGGCTGCAAAC	TCTGGGGTCCTAGGGAATTG
NCAM	NM_000615.6	ACTCTCCAACGCTGATCTCC	CAGCCAGCAGATTACAATGC
TUJ1	NM_001197181.1	GCTCAGGGGCCTTTGGACATCTCTT	TTTTCACACTCCTTCCGCACCACATC
polycistronic *OKSIM*	N/A	GACCACCTCGCCTTACACAT	TTCAGCTCCGTCTCCATCAT
FOXA2	NM_021784.4	TACGTGTTCATGCCGTTCAT	CGACTGGAGCAGCTACTATGC
BRACHYURY	NM_003181.3	CCCTATGCTCATCGGAACAA	CAATTGTCATGGGATTGCAG

Table S2. Table of qRT-PCR primer sequences.

Supplemental Movie (separate file):

Movie S1. Related to Figure 6. hiNSCs demonstrate functional calcium signaling in a 3D brain model. Video of live calcium imaging (using the Fluo4AM calcium indicator) of hiNSCs cultured in 3D brain donut model for 3 weeks. Video shows spontaneous firing between neurons.

Supplemental Experimental Procedures:

Polycistronic lentivirus production for reprogramming
To generate pluripotent stem cells from somatic cells, a vector that expresses the reprogramming factors OCT4, KLF4, SOX2, and cMYC in a polycistronic lentivirus was used (Addgene #24603, a gift from Jose Cibelli). This polycistronic lentivirus also contains a LoxP site that allows for transgene removal upon addition of Cre-recombinase. HEK293 cells were used for packaging the virus. These cells were grown in DMEM, 10% fetal bovine serum (FBS), and 1% antibiotic-antimycotic. The cells were co-transfected with the lentivirus construct, and psPAX and pMD2.G packaging vectors (Addgene #12260 and 12259, gifts from Didier Trono) using Fugene (Roche). Culture medium was harvested 24- and 48-hrs post-transfection. Viral particles were concentrated using Lenti-X concentrator (Clontech), then centrifuged at 3000 rpm. Concentrated viruses were titered and subsequently stored at -80°C until further use.

Pluripotent stem cell and neural stem cell culture
The H1 human ES cell line (Wicell) was grown on mouse embryonic fibroblast feeder layers inactivated by mitomycin C treatment in Knockout (KO) DMEM supplemented with 20% KO serum replacement (not xeno-free), 10 ng/mL recombinant bFGF, 1% Glutamax, 1% antibiotic-antimycotic, and 0.1 mM β-mercaptoethanol (Invitrogen). Colonies were expanded by enzymatically passaging using TrypLE (Invitrogen). Commercially available cell lines were cultured as controls for human neural stem cells. H9-NSCs (Invitrogen) are neural stem cells derived from H9 hESC lines. Human neural progenitors (hNPs, ReNcell) are an immortalized human neural progenitor cell line derived from human fetal brain tissue. Both H9-NSCs and hNPs were cultured using hiNSC media, on gelatin and laminin (Roche) coated plates, respectively.

Differentiation into neuronal and glial phenotypes
For spontaneous differentiation, hiNSC colonies were enzymatically removed from MEF feeder layers using TrypLE (Invitrogen), then dissociated by manual pipetting. Larger aggregates were removed from cell suspension using a 40-70 μM cell strainer. Dissociated hiNSCs were plated and cultured on multiple substrates including laminin (Roche) or gelatin in hiNSC basal media without bFGF or in Neurobasal media supplemented with 2% B27 (Invitrogen), 1% Glutamax, and 1% antibiotic-antimycotic. For guided differentiation, Shh (500 ng/ml, Peprotech), FGF8 (500 ng/ml, Peprotech), and RA (R&D) at concentrations between 10-100 μM were used to facilitate neuronal and glial differentiation within shorter culture periods (~7-8 days). For differentiation studies in undefined media, DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic was used.

Co-culture of hiNSCs with skeletal muscle cells
C2C12 murine myoblast cell line (ATCC) was cultured in DMEM+10%FBS (Invitrogen). For skeletal muscle differentiation, cells were cultured at high confluence in DMEM+1%FBS to induce myotube formation. hiNSC colonies that had been removed from feeders and subcultured on gelatin in hiNSC media (without bFGF) for 2 weeks were subsequently trypsinized with TrypLE, then passaged onto differentiating C2C12 cultures. Co-cultures were maintained for 4-5 days, and fixed for immunofluorescent analysis.

3D cortical brain tissue model
A 3D cortical brain tissue model was generated as previously described (*17, 18*). Briefly, porous, silk protein sponges were generated and biopsy punched into 6 mm discs,with 2 mm holes in the center to form donut shaped scaffolds. These scaffolds were autoclaved and coated with laminin (0.5 mg/ml) (Roche, Indianapolis, IN). Dissociated hiNSCs were seeded into the scaffolds at a density of $1x10^6$ and allowed to adhere overnight. The following day, collagen gels were made using rat tail collagen (Corning, Bedford, MA, USA) as previously described. 3D tissue models were then cultured in neurobasal media (Invitrogen, Carlsbad, CA) supplemented with 2% B27 (Invitrogen, Carlsbad, CA), 0.5 mM Glutamax, and 1% antibiotic-antimycotic (Invitrogen, Carlsbad, CA) for up to 12 weeks, with media changes every 3 days.

Immunofluorescence
Briefly, cells grown in culture plates, on coverslips, or in 3D silk scaffold cultures were fixed in 4% paraformaldehyde, then washed with 1X phosphate-buffered saline (PBS). Samples were incubated with blocking buffer consisting of PBS containing 10% goat serum and 0.1% triton X-100. Primary antibodies were added to blocking buffer, and incubated with samples overnight at 4ºC. The following day, samples were washed several times with PBS, then incubated with a corresponding fluorescently-conjugated secondary antibody in blocking

buffer, for 1 hour at room temperature (away from light). Nuclei were counterstained with DAPI (Invitrogen). For immunostaining samples from *in vivo* studies, 4% paraformaldehyde-fixed cryosectioned tissues were used following a similar immunostaining protocol. All antibodies used in this study are listed in Table S1.

qRT-PCR
Total RNA was isolated using the RNeasy Mini kit (Qiagen), and cDNA was generated using MLV-reverse transcriptase (Invitrogen, CA) according to the manufacturers' protocols. Quantitative RT-PCR was performed on the iQ5 Real-Time PCR Detection System (BioRad) and normalized against the housekeeping gene GAPDH. All primer sequences are listed in Table S2.

Calcium imaging
Cells plated onto coverslips or on 3D scaffolds were immersed in extracellular solution (NaCl 140 mM, KCl 2.8 mM, CaCl2 2 mM, MgCl2 2 mM, HEPES 10 mM, glucose 10 mM, pH = 7.4, adjusted with NaOH). Fluo-4 (Invitrogen) calcium sensitive dye was mixed 1:1 with 20% Pluronic F127 (Invitrogen). Next, Fluo-4 was diluted to a final concentration of 1 μM in the extracellular buffer and pre-warmed to 37°C. The Fluo-4 1 μM solution was applied to cells and incubated at 37°C for 1 hour. Upon incubation, cells were washed with the extracellular buffer to remove any unreacted dye. Cells were imaged using Olympus MVX10 macroscope (12.6x magnification) and Hamamatsu ORCA-Flash4.0 camera. The images were taken with the following setup: 15 ms exposure, 60 ms frame frequency, 512x512 pixel, 4x4 binning, 1000 frames/minute over 3 minutes at room temperature. Some samples were treated with 200 μM picrotoxin (Sigma-Aldrich) just before imaging. The movie was created using ImageJ software (NIH).

Electrophysiology
hiNSCs were grown on poly-L-lysine coated coverslips (Corning) in Neurobasal media supplemented with 2% B27, 1% Glutamax, and 1% antibiotic-antimycotic for 1-8 weeks. Whole cell patch clamp technique was used for all recordings using glass electrodes (3-6 MΩ) and were performed at 34°C. Pipette saline contained (in mM): 130 K-gluconate, 10 KCl, 0.1 $CaCl_2$, 2 Mg-ATP, 1.1 EGTA, and 10 HEPES, pH 7.4 KOH. Bath saline contained the following (in mM): 140 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES, and 11 glucose, pH 7.4 NaOH. To determine the functionality of $GABA_A$ receptors, muscimol (1μM) was applied onto 1-week old hiNSC using a three-barreled 700 μm pipe positioned next to the cell (Warner Instruments), and responses were recorded in current clamp (I=0). To determine if 8 week old hiNSCs were capable of generating action potentials, we performed current clamp experiments (1-second duration, 20 pA steps, covering a range from -100 pA to +300 pA). To assess the ability of hiNSCs to generate spontaneous action potentials, recordings were made in current clamp (I=0 mode). All data were obtained with an Axopatch 200B amplifier and pclamp 10 software (Molecular Devices LLC, Sunnyvale, CA, USA), and analyzed offline using Clampfit. All recordings were digitized at 10 kHz.

Injection of hiNSCs into chick embryos
hiNSCs were enzymatically removed from MEF feeder layers using TrypLE (Invitrogen), and subsequently dissociated by manual pipetting to achieve a single cell suspension. Cells were then fluorescently labeled using DiD (Invitrogen) and washed repeatedly to remove excess dye. Hamburger Hamilton Stage 16 (~55 hours of incubation) chicken embryos (UConn) were used. Briefly, a small window was made in the eggshell to access the embryo, and PBS with antibiotic-antimycotic was added to prevent infection. Fast green dye (1 μl) was added to the cell suspension to visualize the location of the injected cells. Cells entered a pulled borosilicate glass needle by capillary action, and were subsequently injected into the lumen of the developing chick neural tube using a micromanipulator (Parker Picospritzer II). The windowed egg was then sealed using tape. Embryos were harvested and fixed with 4% paraformaldeyde between 1-8 days post-injection for subsequent analysis. Embryos to be cryosectioned were first equilibrated in 15% sucrose-PBS solution, then embedded in OCT. Sections of 10 μM thickness were prepared on slides using a cryostat (Leica).

Microscopy
Brightfield and fluorescent images were obtained using a Keyence BZ-X700 microscope and associated software. Images of 3D fluorescence were taken using confocal or two photon excited fluorescence (TPEF) using a Leica (Wetzlar, Germany) DMIRE2 microscope with a TCS SP2 scanner. Live calcium imaging was performed using Olympus MVX10 macroscope.

Statistics
All data are expressed as mean ± SD, with at least 3 independent samples analyzed per experiment. Data

demonstrating any statistically significant differences were determined by 1-factor ANOVA with post-hoc Tukey test using the statistics software SYSTAT12 (Systat). A p-value less than 0.05 was considered significant.