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Supplemental Information

A Robust Single Primate Neuroepithelial Cell Clonal Expansion System for Neural Tube Development and Disease Studies

Xiaoqing Zhu, Bo Li, Zongyong Ai, Zheng Xiang, Kunshang Zhang, Xiaoyan Qiu, Yongchang Chen, Yuemin Li, Joshua D. Rizak, Yuyu Niu, Xintian Hu, Yi Eve Sun, Weizhi Ji, and Tianqing Li

Supplemental Figures

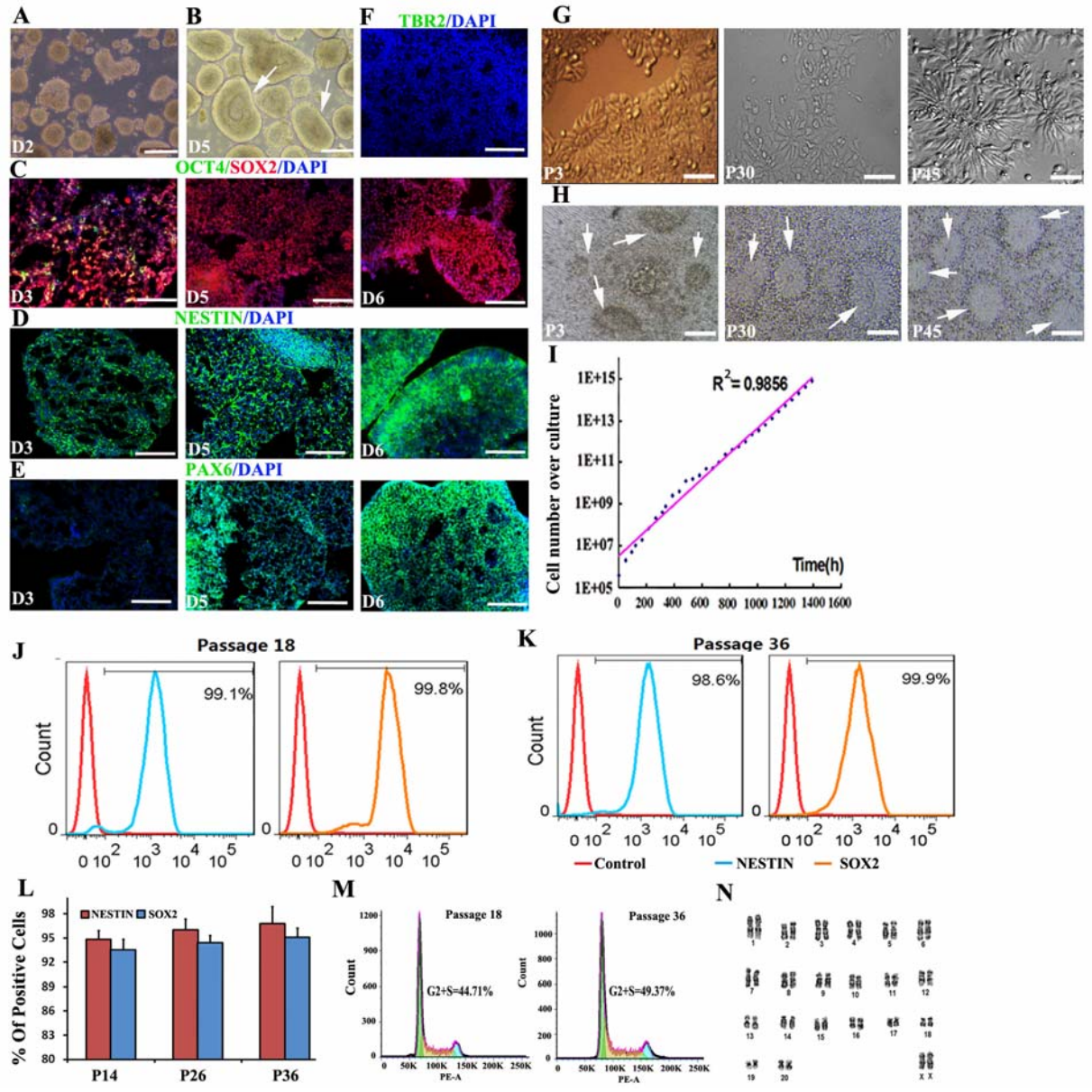

Figure S1.

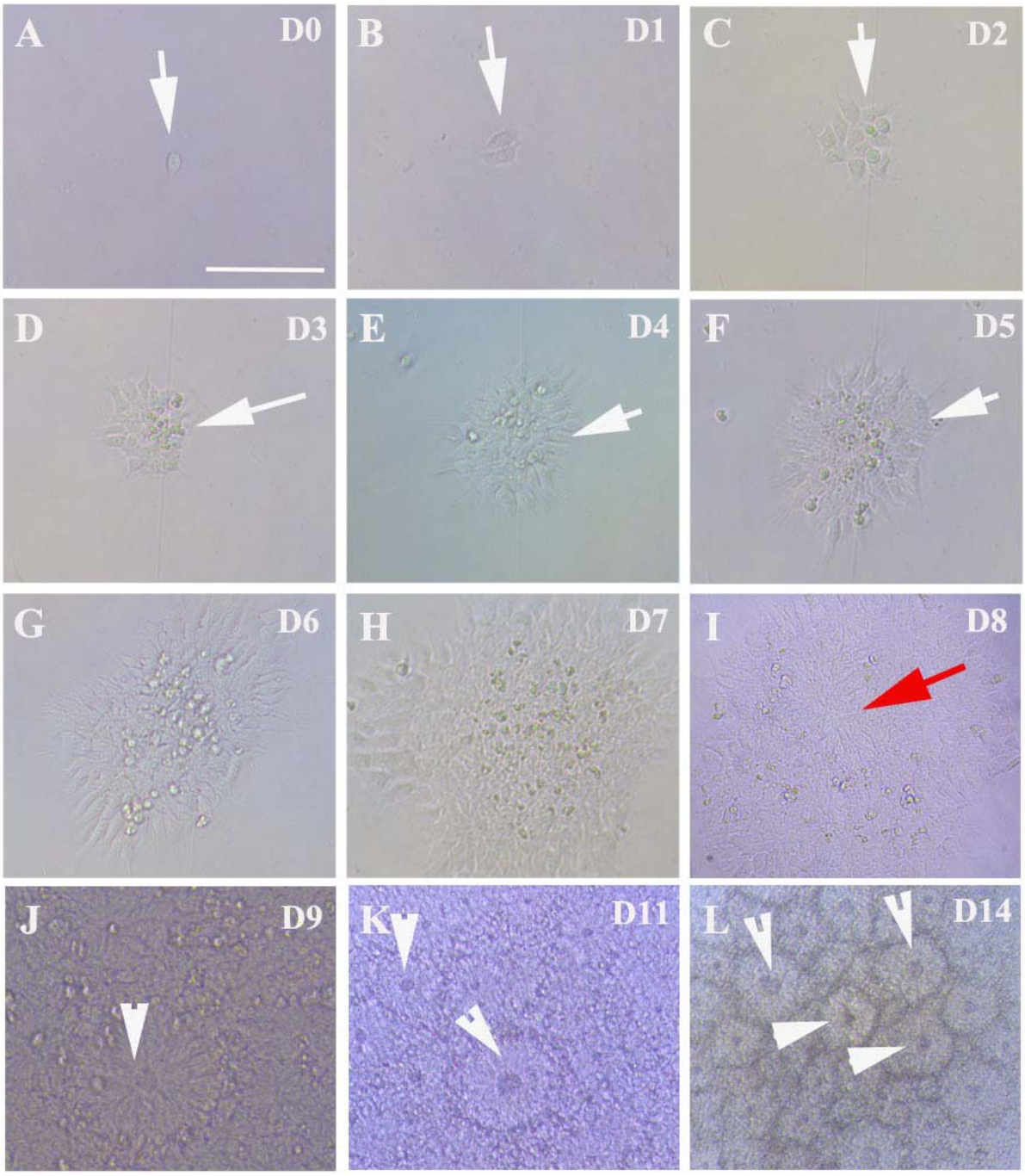

Figure S2

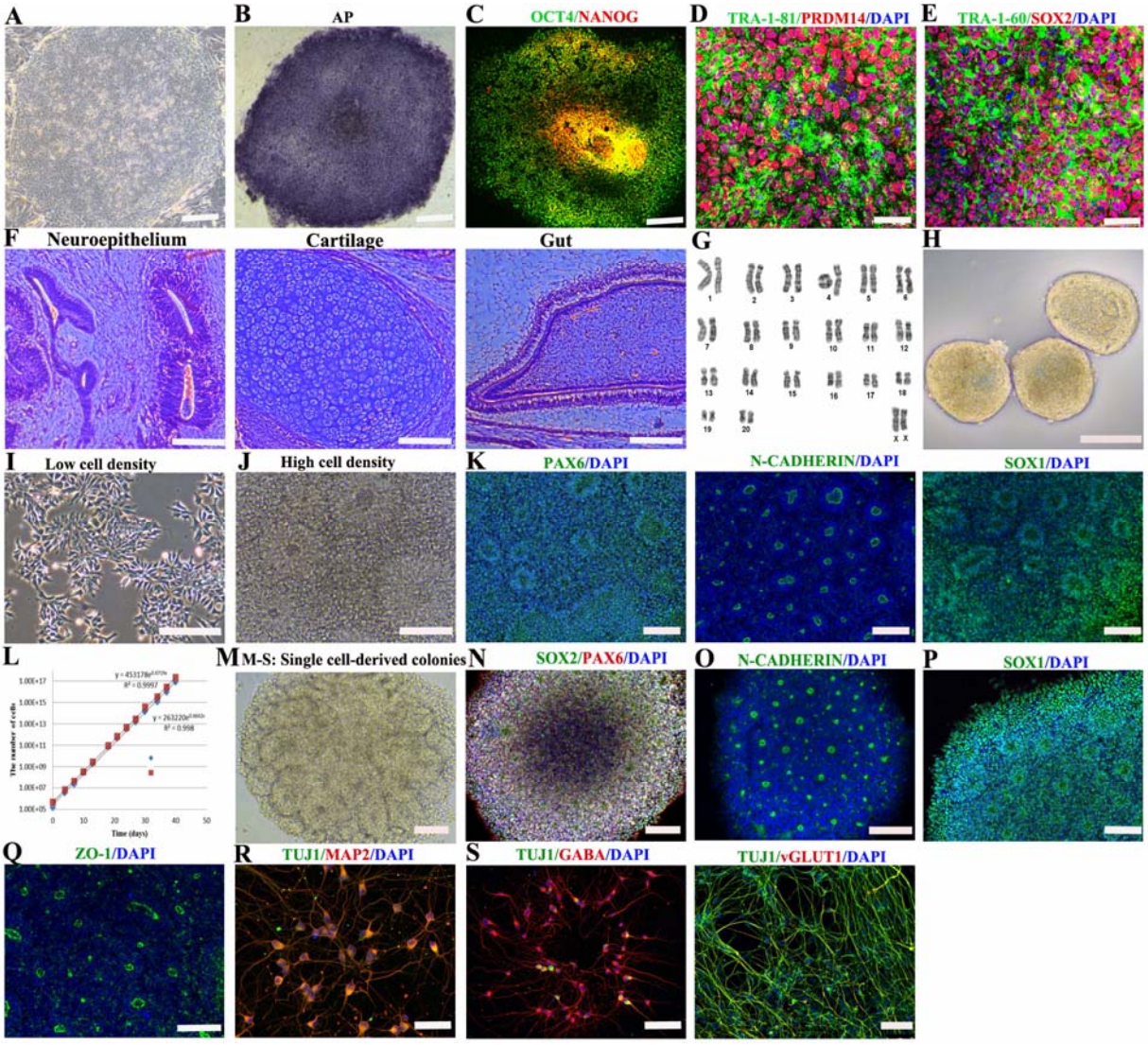
A
B
AP
C
OCT4/NANOG
D
TRA-1-81/PRDM14/DAPI
E
TRA-1-60/SOX2/DAPI
F
Neuroepithelium
Cartilage
Gut
G
H
I
Low cell density
J
High cell density
K
PAX6/DAPI
N-CADHERIN/DAPI
SOX1/DAPI
L
M
M-S: Single cell-derived colonies
N
SOX2/PAX6/DAPI
O
N-CADHERIN/DAPI
P
SOX1/DAPI
Q
ZO-1/DAPI
R
TUJ1/MAP2/DAPI
S
TUJ1/GABA/DAPI
TUJ1/vGLUT1/DAPI

Figure S3

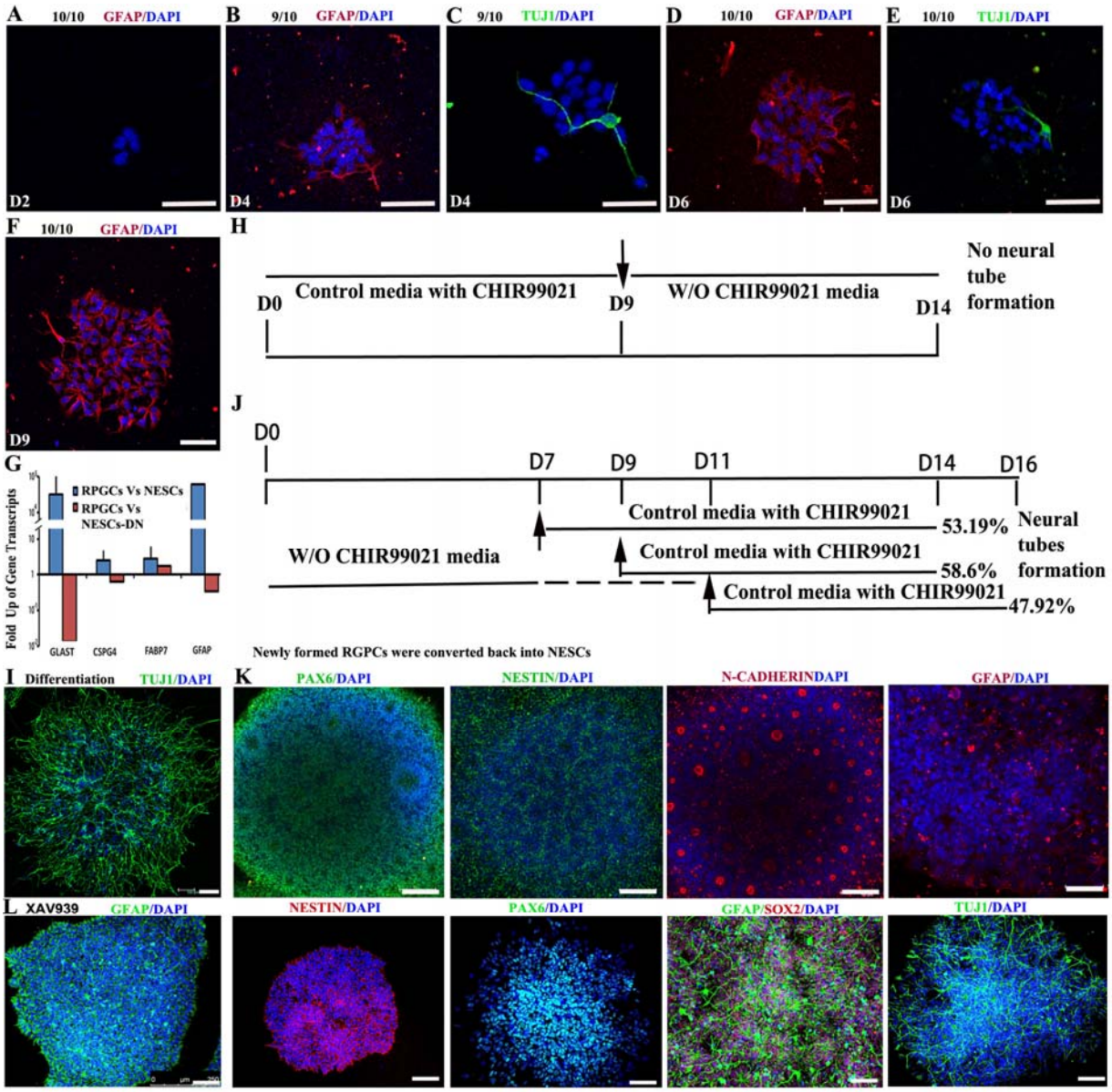
A 10/10 GFAP/DAPI
D2
B 9/10 GFAP/DAPI
D4
C 9/10 TUJ1/DAPI
D4
D 10/10 GFAP/DAPI
D6
E 10/10 TUJ1/DAPI
D6
F 10/10 GFAP/DAPI
D9
H
Control media with CHIR99021
W/O CHIR99021 media
D0
D9
D14
No neural tube formation
J
D0
D7
D9
D11
D14
D16
Control media with CHIR99021
53.19%
W/O CHIR99021 media
Control media with CHIR99021
58.6%
Control media with CHIR99021
47.92%
Neural tubes formation
G
RPGCs Vs NESCs
RPGCs Vs NESCs-DN
Fold Up of Gene Transcripts
GLAST
CSPG4
FABP7
GFAP
Newly formed RGPCs were converted back into NESCs
I Differentiation TUJ1/DAPI
K PAX6/DAPI
NESTIN/DAPI
N-CADHERINDAPI
GFAP/DAPI
L XAV939 GFAP/DAPI
NESTIN/DAPI
PAX6/DAPI
GFAP/SOX2/DAPI
TUJ1/DAPI

Figure S4.

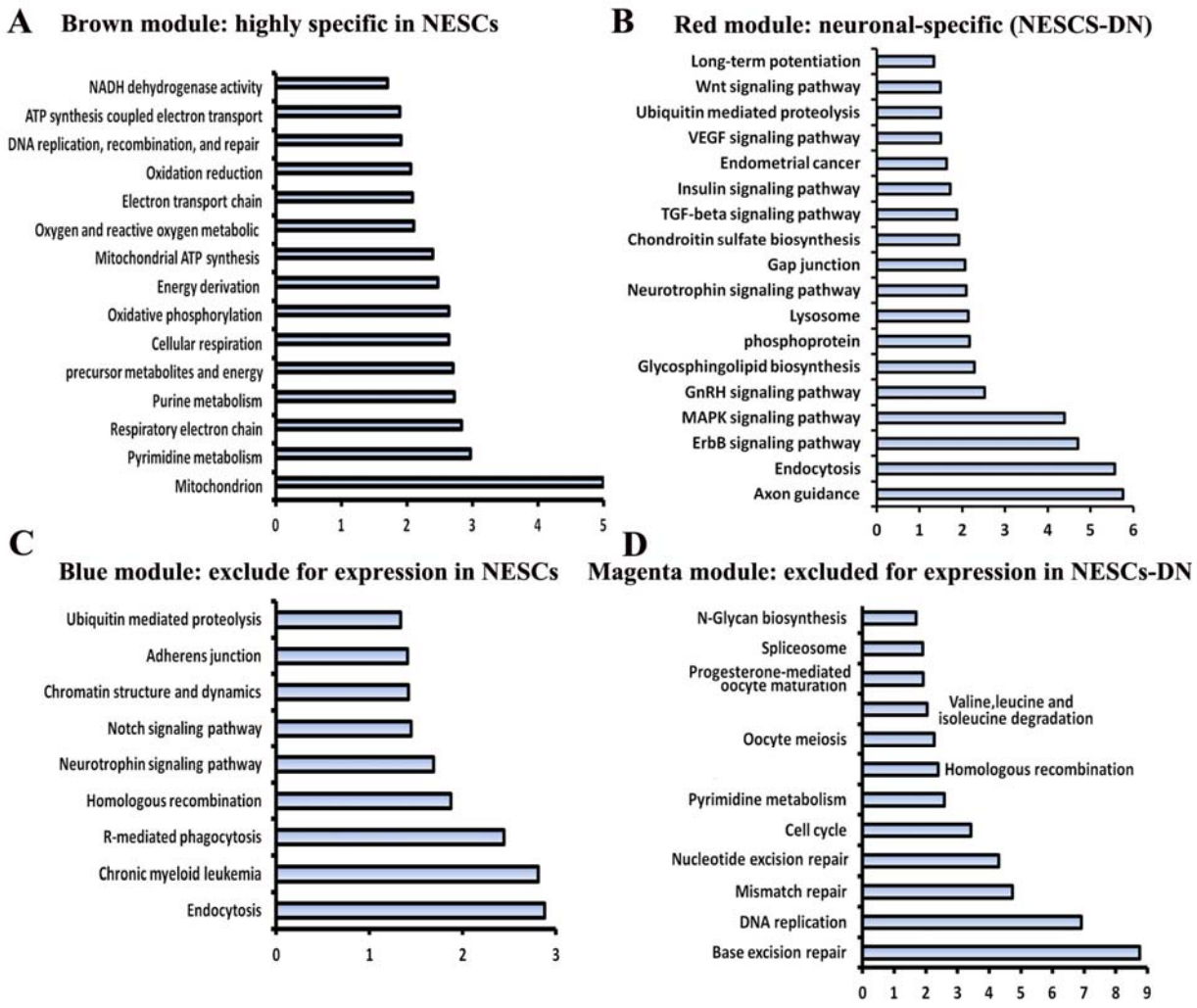
A
Brown module: highly specific in NESCs
NADH dehydrogenase activity
ATP synthesis coupled electron transport
DNA replication, recombination, and repair
Oxidation reduction
Electron transport chain
Oxygen and reactive oxygen metabolic
Mitochondrial ATP synthesis
Energy derivation
Oxidative phosphorylation
Cellular respiration
precursor metabolites and energy
Purine metabolism
Respiratory electron chain
Pyrimidine metabolism
Mitochondrion
0 1 2 3 4 5
B
Red module: neuronal-specific (NESCS-DN)
Long-term potentiation
Wnt signaling pathway
Ubiquitin mediated proteolysis
VEGF signaling pathway
Endometrial cancer
Insulin signaling pathway
TGF-beta signaling pathway
Chondroitin sulfate biosynthesis
Gap junction
Neurotrophin signaling pathway
Lysosome
phosphoprotein
Glycosphingolipid biosynthesis
GnRH signaling pathway
MAPK signaling pathway
ErbB signaling pathway
Endocytosis
Axon guidance
0 1 2 3 4 5 6
C
Blue module: exclude for expression in NESCs
Ubiquitin mediated proteolysis
Adherens junction
Chromatin structure and dynamics
Notch signaling pathway
Neurotrophin signaling pathway
Homologous recombination
R-mediated phagocytosis
Chronic myeloid leukemia
Endocytosis
0 1 2 3
D
Magenta module: excluded for expression in NESCs-DN
N-Glycan biosynthesis
Spliceosome
Progesterone-mediated oocyte maturation
Valine,leucine and isoleucine degradation
Oocyte meiosis
Homologous recombination
Pyrimidine metabolism
Cell cycle
Nucleotide excision repair
Mismatch repair
DNA replication
Base excision repair
0 1 2 3 4 5 6 7 8 9

Figure S5.

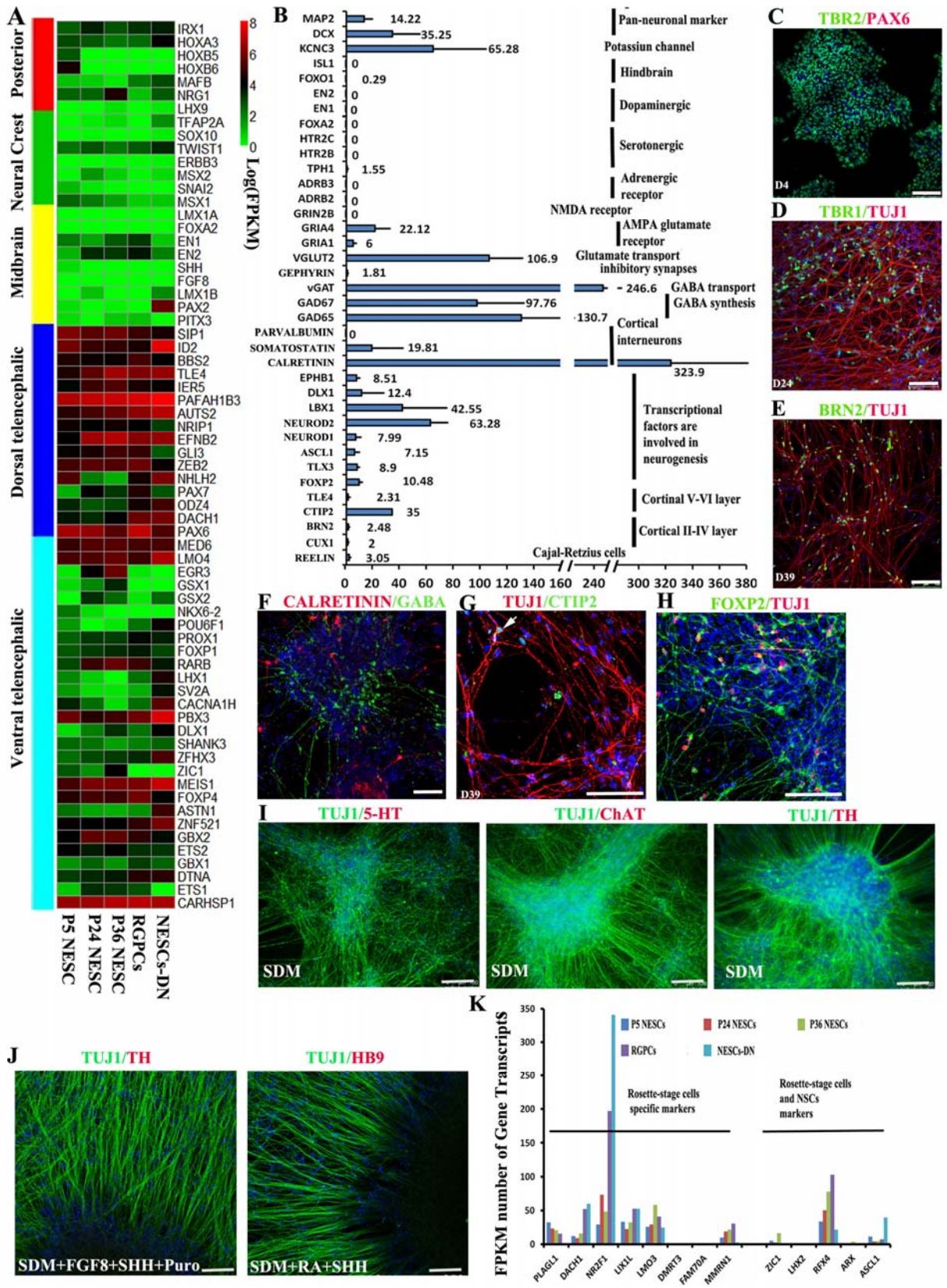
A
Posterior
Neural Crest
Midbrain
Dorsal telencephalic
Ventral telencephalic
IRX1
HOXA3
HOXB5
HOXB6
MAFB
NRG1
LHX9
TFAP2A
SOX10
TWIST1
ERBB3
MSX2
SNAI2
MSX1
LMX1A
FOXA2
EN1
EN2
SHH
FGF8
LMX1B
PAX2
PITX3
SIP1
ID2
BBS2
TLE4
IER5
PAFAH1B3
AUTS2
NRIP1
EFNB2
GLI3
ZEB2
NHLH2
PAX7
ODZ4
DACH1
PAX6
MED6
LMO4
EGR3
GSX1
GSX2
NKX6-2
POU6F1
PROX1
FOXP1
RARB
LHX1
SV2A
CACNA1H
PBX3
DLX1
SHANK3
ZFHX3
ZIC1
MEIS1
FOXP4
ASTN1
ZNF521
GBX2
ETS2
GBX1
DTNA
ETS1
CARHSP1
8
6
4
2
0
Log(FPKM)
P5 NESC
P24 NESC
P36 NESC
RGPCs
NESCs-DN
B
MAP2 14.22
DCX 35.25
KCNC3 65.28
ISL1 0
FOXO1 0.29
EN2 0
EN1 0
FOXA2 0
HTR2C 0
HTR2B 0
TPH1 1.55
ADRB3 0
ADRB2 0
GRIN2B 0
GRIA4 22.12
GRIA1 6
VGLUT2 106.9
GEPHYRIN 1.81
vGAT 246.6
GAD67 97.76
GAD65 130.7
PARVALBUMIN 0
SOMATOSTATIN 19.81
CALRETININ 323.9
EPHB1 8.51
DLX1 12.4
LBX1 42.55
NEUROD2 63.28
NEUROD1 7.99
ASCL1 7.15
TLX3 8.9
FOXP2 10.48
TLE4 2.31
CTIP2 35
BRN2 2.48
CUX1 2
REELIN 3.05
Pan-neuronal marker
Potassiun channel
Hindbrain
Dopaminergic
Serotonergic
Adrenergic receptor
NMDA receptor
AMPA glutamate receptor
Glutamate transport inhibitory synapses
GABA transport
GABA synthesis
Cortical interneurons
Transcriptional factors are involved in neurogenesis
Cortinal V-VI layer
Cortical II-IV layer
Cajal-Retzius cells
0 20 40 60 80 100 120 140 160 240 300 320 340 360 380
C TBR2/PAX6
D4
D TBR1/TUJ1
D24
E BRN2/TUJ1
D39
F CALRETININ/GABA
G TUJ1/CTIP2
D39
H FOXP2/TUJ1
I TUJ1/5-HT
TUJ1/ChAT
TUJ1/TH
SDM
SDM
SDM
J TUJ1/TH
TUJ1/HB9
SDM+FGF8+SHH+Puro
SDM+RA+SHH
K
FPKM number of Gene Transcripts
350
300
250
200
150
100
50
0
P5 NESCs
P24 NESCs
P36 NESCs
RGPCs
NESCs-DN
Rosette-stage cells specific markers
Rosette-stage cells and NSCs markers
PLAGL1
DACH1
NR2F1
LIX1L
LMO3
DMRT3
FAM70A
MMRN1
ZIC1
LHX2
RFX4
ARX
ASCL1

Figure S6.

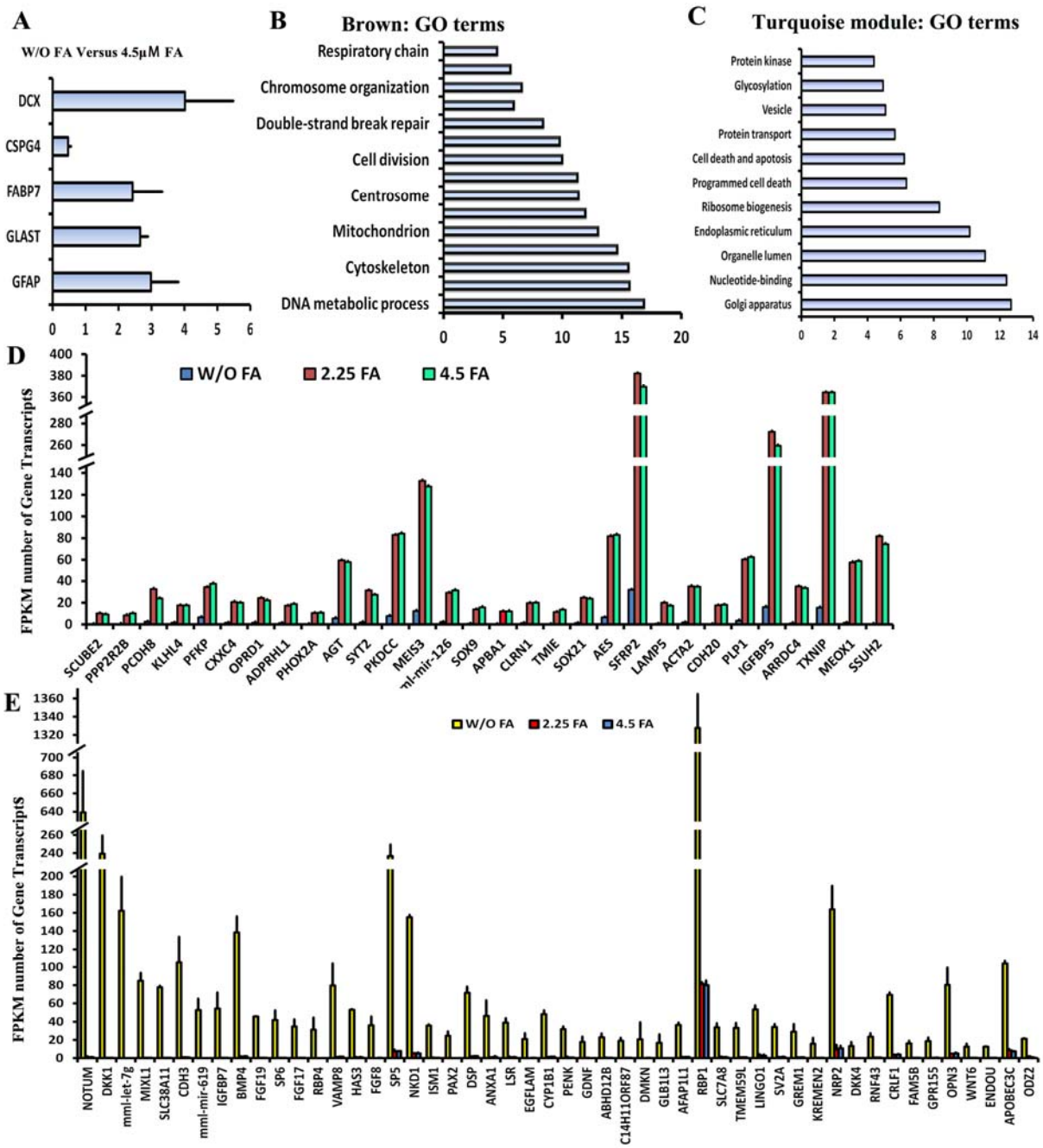
A
W/O FA Versus 4.5μM FA
DCX
CSPG4
FABP7
GLAST
GFAP
0 1 2 3 4 5 6
B
Brown: GO terms
Respiratory chain
Chromosome organization
Double-strand break repair
Cell division
Centrosome
Mitochondrion
Cytoskeleton
DNA metabolic process
0 5 10 15 20
C
Turquoise module: GO terms
Protein kinase
Glycosylation
Vesicle
Protein transport
Cell death and apotosis
Programmed cell death
Ribosome biogenesis
Endoplasmic reticulum
Organelle lumen
Nucleotide-binding
Golgi apparatus
0 2 4 6 8 10 12 14
D
W/O FA
2.25 FA
4.5 FA
FPKM number of Gene TranscriptS
SCUBE2 PPP2R2B PCDH8 KLHL4 PFKP CXXC4 OPRD1 ADPRHL1 PHOX2A AGT SYT2 PKDCC MEIS3 SOX9 APBA1 CLRN1 TMIE SOX21 AES SFRP2 LAMP5 ACTA2 CDH20 PLP1 IGFBP5 ARRDC4 TXNIP MEOX1 SSUH2
E
W/O FA
2.25 FA
4.5 FA
FPKM number of Gene TranscriptS
NOTUM DKK1 MIXL1 SLC38A11 CDH3 IGFBP7 BMP4 FGF19 SP6 FGF17 RBP4 VAMP8 HAS3 FGF8 SP5 NKD1 ISM1 PAX2 DSP ANXA1 LSR EGFLAM CYP1B1 PENK GDNF ABHD12B C14H11ORF87 DMKN GLB1L3 AFAP1L1 RBP1 SLC7A8 TMEM59L LINGO1 SV2A GREM1 KREMEN2 NRP2 DKK4 RNF43 CRLF1 FAM5B GPR155 OPN3 WNT6 ENDOU APOBEC3C ODZ2

Figure S7

SUPPLEMENTAL FIGURE LEGENDS

Figure S1, related to Figure 1. Rhesus monkey ES cells (rESCs) are rapidly converted into neuroepithelial stem cells (NESCs) with stable neural tube (NT) formation and neurogenic ability in large-scale expansion. Three independent experiments were repeated for NESCs from IVF3.2 and 3.3, respectively. (A) Typical EBs at post-differentiation Day 2 (pdD2); (B) EBs were organized into a neuroepithelial layer structure at pdD5. (C-E) On pdD2, 5 and 6, EBs were cryosectioned and stained with OCT4, SOX2, NESTIN and Pax6, respectively. (F) NESCs did not express TBR2, a marker of OSVZ and ISVZ progenitors in the human and primate developing cortex. These staining were independently repeated for three times. (G) Rosette structures were formed when cells were continually cultured at low cell density through Passage 3 (P3), 30 (P30) and 45 (P45). (H) The NT structures were maintained when cells were continually cultured at high cell density through P3, P30 and P45. Arrows indicate NTs. (I) The growth curve of NESCs, displaying the prospect of exponential growth over serial passages. NESCs were routinely passaged to 1:8 to 1:16 ratios every 3-4 days. (J-K) FACS data show SOX2 and NESTIN positive cells are similarly distributed and highly enriched in P18 and P36 NESCs populations. (L) Quantification of SOX2 and NESTIN positive cells in P14, P26 and P36 NESCs, respectively. Data expressed as mean±s.t.d (Data from three independent experiments) ($P>0.05$ by Student's t-test). (M) Cell cycle staining and FCAS of P18 and P36 NESCs revealed a stable population of cells in S-phase during the large-scale expansion. (N) NESCs with at least 96 population doublings display normal karyotype. Scale bar: 100μm.

Figure S2, related to Figure 2. Single neuroepithelial stem cell self-organized into neural tubes within 14 days. White arrows indicate single cell-derived colonies. On day 8, neural polarized structure began to form (red arrow). From Day 9, neural tube structure began to self-organization (white arrowheads).

Figure S3, related to Figure 1 and 2. NTs can be generated from monkey iPSCs-derived NESCs in a similar manner. Rhesus monkey skin fibroblasts from embryonic day 65 were infected with the cocktail retroviruses including *SOX2*, *OCT4*, *KLF4* and *C-MYC*. Three days after infection, fibroblasts were digested into single cells and cultured on MEFs in ESC growth media for 10-14 days. Typical iPSC colonies were picked up and subjected for passaging. iPS1.1 cell line was randomly used for pluripotent characterization and neural induction. (A) The morphology of monkey iPSCs cultured on MEFs. (B-E) Monkey iPSCs expressed

pluripotent markers, such as AP (alkaline phosphatase), OCT4, NANOG, TRA-1-81, PRDM14, TRA-1-60 and SOX2. (F) Monkey iPSCs were able to differentiate into tissues of all three germ layers, including ectoderm (neuroepithelium), mesoderm (cartilage) and endoderm (gut), when injected into SCID mouse. (G) The iPS1.1 maintained normal karyotype. (H) The morphology of monkey iPSCs-derived neural bodies at Day 5 in suspension culture. (I) The morphology of cultured Passage 5 iPSCs-NESCs at low cell density. (J) The morphology of cultured Passage 5 iPSCs-NESCs at high cell density. (K) Long-term cultured iPSCs-NESCs at passage 6 expressed stem cell markers: SOX2, PAX6, N-CADHERIN and SOX1. (L) Growth curve of iPSCs-NESCs, displaying the prospect of exponential growth over serial passages. (M-Q) Single iPSCs-NESCs self-organized into neural tubes expressing PAX6, N-CADHERIN, NESTIN and ZO-1. (R) Long-term cultured single iPSCs-NESCs gave rise to $MAP2^{+}TUJ1^{+}$ neurons. (S) Differentiated neurons from single iPSCs-NESCs were glutamergic and GABAergic neurons. Scale bars: A-C and F-G, 200 μm; D-E, 50 μm; others, 100 μm. The experiment was independently repeated for three times.

Figure S4, related to Figure 4 and 5. CHIR99021 removal resulted in rapid radial glial progenitor cell (RGPC) transition from NESCs, however, its treatment promoted newly formed RGPCs go back to the NESC state and form neural tubes. (A-H) CHIR99021 removal resulted in rapid "NESC-to-RGPC" transition. (A-F) All progenies of single NESCs were progressively converted to most of GFAP positive cells and few $TUJ1^{+}$ cells in media without CHIR99021. 10(n)/10(m) indicated that 10(n) colonies in all examined 10(m) colonies have identical phenotypes represented by the picture. (G) RNA-seq data showed NESCs in media without CHIR99021 upregulated expression of RGPC markers including *GLAST*, *CSPG4*, *FABP7* and *GFAP* at day 7. Data are from two independent experiments. (H) Experimental paradigm of CHIR99021 treatments. The CHIR99021 was added in the media from Day 0-9 (early-addition), followed by growth in media without CHIR99021 from Day 9-14 (late-withdrawal). The late withdrawal of CHIR99021 resulted in the total loss of NT formation. (I) Formed RGPCs gave rise into $TUJ1^{+}$ neurons once differentiations were induced. (J-K) Newly formed RGPCs can go back to the NESC state and form NTs upon CHIR99021 treatment. (J) Experimental paradigm of early-withdrawal (from Day 0-7, 0-9, or 0-11) followed by late-addition (from Day 7-14, 9-14, or 11-16) of CHIR99021 into the culture media. Single cells were cultured in media W/O CHIR99021 for different periods. (K) Reversed NESCs expressed PAX6, NESTIN, and N-CADHERIN, but not GFAP. (L) Addition of XAV939, a Wnt inhibitor, also completely inhibited NT formation and converted NESCs into RGPCs. Scale bar: A-F, 50μm; others, 100μm.

The experiment was independently repeated for three times.

Figure S5, related to Figure 4. NESCs have unique gene expression profiles. Gene ontology (GO) analysis for genes from different modules in Figure 4B.

Figure S6, related to Figure 4. NESCs were regionally restricted into telencephalic fate and gave rise to cortical neurons. (A) Heatmap showed NESCs and differentiated neurons highly express dorsal and ventral telencephalic markers, but negative for midbrain, neural crest and hindbrain (posterior) markers. (B) Fold upregulation changes of neuronal marker gene transcription levels in NESCs-derived differentiated neurons versus NESCs via RNA sequencing analysis. Data were from two independent experiments. (C) NESCs differentiated into $TBR2^{+}PAX6^{-}$ intermediate progenitors. (D) NESCs differentiated into $TBR1^{+}$ cortical neurons. (E-F) NESCs differentiated into II-IV upper layer $BRN2^{+}$ and $CALERETININ^{+}GABA^{-}$ cortical neurons. (G-H) NESCs differentiated into V-VI upper layer $CTIP2^{+}$ and $FOXP2^{+}$ cortical neurons. (I) NESCs were unable to differentiate into 5-HT, ChAT and TH dopamine neurons in SDM media. (J) NESCs have inability to give rise to TH dopamine neurons and HB9 motoneurons even in the specific dopamine neuron and motoneuron differentiation media. (K) Genes of published rosette-stage cells and $NSCs^{EGF/FGF}$ low or negatively expressed in NESCs, RGPCs and differentiated neurons. Scale bars: 100μm.

Figure S7, related to Figure 6 and 7. The mechanisms of NTDs caused by folic acid (FA) deficiency. (A) Down fold of expression levels of radial glial progenitor cell (RGPCs) markers in NESCs cultured in the media without FA, compared with in the media with FA. (B) Representative GO function terms of FA addition secondly upregulated brown module in Figure 6G. (C) Representative GO function terms of FA deficiency secondly upregulated brown module in Figure 6G. (D) Some representative upregulated genes induced by FA. (E) Some representative upregulated genes of NESCs in absence of FA. The data in A, D and E are two independent experiments.

SUPPLEMENTAL VIDEO LEGENDS

Video S1: A representative 3D reconstruction of a single cell-derived neural tube. Self-organized neural tubes by single NESCs were fixed and stained with DAPI (blue) on Day 14. A single cell-derived neural tube was scanned using a Leica TCS SP8 confocal laser scanning system. Ten neural tubes were scanned.

Video S2: Interkinetic nuclear migration (IKNM) in single cell-derived neural tubes. Live imaging of retrovirus GFP infected NESCs revealed movement of nuclei along apical and basal surface. Arrows mark two NESCs in particular with clear IKNM. Horizontal divisions (0-30°, spindle orientation) were close to apical side in NTs. Time shown in hrs: min.

SUPPLEMENTAL TABLE LEGENDS

Table S1

The gene list of GO terms for different module in supplemental Figure 5.

Table S2

The gene list of GO terms for different module when NESCs cultured in the media without folic acid, or with 2.25 μM or 4.5 μM folia acid, respectively. The GO terms are relative to Figure 7 and Supplement Figure 6.

SUPLEMENTAL EXPERIMENTAL PROCEDURES

Embryonic stem cell culture and neuroepithelial stem cell (NESC) induction

IVF3.2 and IVF3.3 rhesus monkey embryonic stem cells (rESCs), and monkey fibroblast-derived iPS line 1.1 were cultured on X-ray inactivated CF-1 mouse embryonic fibroblasts (MEFs) in ESCs growth media [DMEM/F12 (1:1) (Invitrogen) containing 15% KSR (Invitrogen) and 5ng/mL bFGF (Millipore)] (Li et al., 2005; Sun et al., 2011). The monkey fibroblast-derived iPS line 1.1 was obtained by the modified protocol (Liu et al., 2008). Briefly, skin fibroblasts were obtained from one male rhesus monkey fetuses of embryonic 65 days. Plasmids pMX-*OCT4*/*SOX2*/*KLF4*/*C-MYC* 6μg, pCL6μg were prepared to transfect 293T cells by the $Ca_3(PO4)_2$ method. 12 hours after transfection, the medium was changed. 48 hours after transfection, virus-containing supernatants were collected, filtered through a 0.45 μm pore-size filter and concentrated by 5-fold for use. The four concentrated retroviruses were mixed to infect monkey fibroblasts which were passaged 24 hours before at 3×10^4 cells per 35mm dish. At the first day after infection (piD1), infected fibroblasts were infected by the four concentrated retroviruses again. About 80% of fibroblasts were routinely infected by any one of OCT4, SOX2, KLF4 or C-MYC viruses. At piD3, infected fibroblasts were digested into single cells and cultured on MEFs in ESCs growth media. 10-14 days after infection, typical iPSC colonies were mechanically picked up and subjected for passaging. One cell line iPS1.1 was randomly used for pluripotent characterizations and the following neural induction.

ESCs or iPS1.1 were digested with Collagenase IV (Gibco) and neural induction was induced by switching from ESC growth media to differentiation media in suspension culture [Advance DMEM/F12 (1:1) (Invitrogen): Neurobasal media (Invitrogen) (1:1 mixture) supplemented with 1xN2 (Invitrogen), 1xB27 (Invitrogen), 10ng/ml bFGF (Millipore), 3μM CHIR99021 (Cellagen technology), 5μM SB431542 (Cellagen technology), 0.2 μM Compound E and 0.1μM LDN193189 (Cellagen technology)]. After 6 days,

EBs were transferred to 5μg/ml laminin (Gibco)-coated plates for attachment culture and the media was switched to NESCs culture media [Neurobasal media including 1xB27, 1xN2, 1XNEAA (Sigma), 1% Glutmax (Sigma), 3 μM CHIR99021, 5μM SB431542, 10ng/ml bFGF, and 1000U/ml hLIF (Millipore)].

Culture and large-scale expansion of NESCs

Neuroepithelial stem cells (6 days after EB differentiation of ESCs) were cultured on 5μg/ml laminin (Gibco) coated plates in NESCs culture media (detailed above). 0.025％ trypsin (Sigma) was used to digest NESCs when passaging to encourage cell propagation. NESCs were routinely passaged to 1:8 to 1:16 ratios every 3-4 days. For NT formation, NESCs were continually cultured 8-10 days before passaging.

NESC differentiation

For spontaneous differentiation, NESCs were cultured on laminin (5μg/ml) and gelatin (0.05%) coated plates in differentiation media (SDM) [Neurobasal supplemented with 1xN2, 1xB27, 1XNEAA and 1% Glutmax]. On Day 6, 10ng/ml BDNF (R&D systems) and 10ng/ml GDNF (R&D systems) were added into the media to induce terminal maturation of neurons. For GABAergic neuron differentiation, 10 ng/ml SHH (R&D Systems) was added into the differentiation media for the first 4 days. On Day 5, 10ng/ml BDNF (R&D systems) and 10ng/ml GDNF (R&D systems) were added into the media to induce terminal maturation of neurons. For density differentiation experiments, the differentiations were induced at $9.5x10^3$ cells/cm^2 for high cell density differentiation and at 2.1 $x10^3$ cells/cm^2 for low cell density differentiation, respectively.

To induce dopaminergic neuron differentiation, NESCs was first cultured in the SDM media including 500ng/ml SHH (R&D Systems), 500 ng/ml FGF8 and 4μM purmorphamine for 7 days, following in the dopamine neuron matured media including 10ng/ml GDNF, 10 ng/ml BDNF, 10ng/ml IGF and 0.5 mM db-cAMP for another 10 days.

For motoneuron differentiation, NESCs were treated by the SDM media surplus with 200 ng/ml SHH and 1μM RA (retinoic acid) for 7 days. To facilitate the maturation of differentiated neurons, the differentiated cells were also treated with 10ng/mL BDNF and 10ng/mL GDNF for additional 2-3 weeks.

Immunocytochemistry and flow cytometry

For immunocytochemistry, cells were fixed with 4% paraformaldehyde for 15 mins, washed with PBS, treated with 0.1% Triton X-100 (Invitrogen) for 20 mins, washed three times with PBS, and incubated in blocking buffer (0.1% Tween 20 and 10% normal donkey serum (Invitrogen) in PBS) for 30 min at room temperature. The cells were incubated with primary antibody overnight at 4 ^{o}C. Primary antibodies are listed in the following: TUJ1 (Covance, MRB435P, 1:1000); TUJ1 (Millipore, MAB1637, 1:1000); TBR2 (Millipore, AB2283, 1:300) ; TH (Millipore, AB152, 1:400); Cholinergic (ChAT) (Millipore, AP144P, 1:600); HB9 (Millipore, ABN174,1:500); SOX2 (Millipore, MAB5603, 1:400); NESTIN (Millipore, MAB5922, 1:400); MAP-2 (Millipore, MAB5622, 1:600); SYNAPSIN I (Sigma, S193, 1:500); GFAP (Sigma, G9269, 1:2000); BrdU (Sigma, B8434, 1:300); GABA (Sigma, A2052, 1:600); vGLUT1 (Sigma, V0389, 1:1000); SEROTONIN (5-HT) (Sigma, S5545, 1:1500); P-VIMENTIN (MBL International, D076-3S, 1:200); PAX6 (RD, MAB1260, 1:1000); SOX1 (R&D, AF3369, 1: 400); PSD95 (ABCAM, ab2723, 1:300); GFP (Abcam, ab13970, 1:1000); OCT4 (Santa Cruz, SC5279, 1:400); N-CADHERIN (Santa Cruz, SC7939, 1:50) and ZO-1 (Invitrogen, 339100, 1:50). The following day, the cells were washed with PBS and incubated with Alexa488 or rhodamine-conjugated secondary antibodies (Invitrogen: goat-anti-rabbit, goat-anti-mouse**,** donkey- anti-goat**,** donkey-anti-chicken, 600×) in PBS for one hour at RT. Nuclei were visualized with DAPI staining (Sigma-Aldrich). For BrdU staining of NTs, NESCs with typical NTs were treated with 5 μ g/ml Bromodeoxyuridine (Sigma) for 0.5 hr (NESC-derived colonies) or 1.5 hr

(single cell-derived colonies) and then fixed with 4% paraformaldehyde. To test the effect of folic acid (FA) on proliferation of NESCs, 5μg/ml BrdU were added into the media for 0.5h after NESCs were cultured in the media without FA, or with 2.25μM FA or 4.5μM FA for three days, respectively. Fixed cells were treated with 0.1% Triton X-100 for 20 min, followed by 1M HCL for 30 min at 45℃.

For indirect flow cytometry, cells were incubated with mouse anti-Nestin and rabbit anti-Sox2 for overnight at 4°C and washed three times with PBS. Cells were then incubated with Fluor 488 or rhodamine conjugated goat anti-rabbit IgG and Fluor 488 or rhodamine conjugated goat anti-mouse IgG (Invitrogen, 1:1,000) for 30 min on ice and washed three times. Cells directly incubated with secondary antibody were used as control. Cell cycle assays were performed following the instructions of the Cell Cycle Kit (Beyotime). Flow cytometry analysis was carried out with a FACScan flow cytometer (BD Biosciences).

RT-PCR

RNA was isolated with Trizol reagent according to the manufacturer's instructions (Invitrogen). cDNA was reserved with the M-MLV first strand kit (Invitrogen) according to the manufacturer's protocol. The primers for *OCT4*, *SOX2*, *PAX6* and the house-keeping gene *GAPDH* are the following: *OCT4*-F, 5'-GTTCAGCCAAACGACCATC-3'; *OCT4*-R, 5'-GGAAAGGGACCGAGGAGTAC-3'; *SOX2*-F, 5'-AGAGTGGAAACTTTTGTCGG-3' , *SOX2*-R, 5'- GGAGTGGGAGGAAGAGGTAA-3'; *PAX6*-F, 5'-CCCGTCCATCTTTGCTTG-3', *PAX6*-R, 5'- TCATAACTCCGCCCATTCA-3'; *GAPDH*-F, 5'-GCATCAACAGCAGCATCGAGAAC-3', *GAPDH*-R, 5'-CTCCATTTGGCCCTTAACTTG-3' .

Single cell clone assays

Single NESCs were diluted to approximately 1000 cells/ml and 1 μl of these cell suspensions were seeded into NESCs media in individual wells of 96-well plate coated with laminin (5 μg/ ml). Wells containing only one cell were confirmed and wells with multiple cells were excluded by visual observation under microscopy 4 hours after seeding. After seeding, the media was replaced with fresh media every 2-3 days until Day 14. The NT colonies were counted under ZO-1 staining, respectively.

Electrophysiology

For electrophysiological recording, matured cells derived from P31 NESCs were cultured on coverslips for 26 days and were placed in a recording chamber continuously superfused with oxygenated artificial cerebral spinal fluid (mACSF) under a Olympus BX50WI upright infrared DIC microscope. The mACSF was composed of 119mM NaCl, 2.5mM KCl, 26mM $NaHCO_3$, 1mM NaH_2PO_4, 11mM Glucose, at pH 7.4, 300 mOsm. Patch clamp recording pipettes with resistance of 4-8 M were pulled using the laser based P-2000 micropipette puller (Sutter Instrument). Whole cell recording was conducted using the Multiclamp 700A amplifier (Molecular Device) with a 10 KHz sampling rate and were low-pass filtered at 1KHz for current clamp recordings. The pipettes were filled with an intracellular solution containing 130mM KCl, 2mM MgCl2, 10mM KOH, 1mM EGTA, 10mM HEPES, 5mM ATP, and 0.3mM GTP (pH 7.2). During current clamping, the current was held to 0 pA (I=0). For current injection steps, ten sweeps were made at each 20 pA increments from -60 to 120 pA. Changes in spike frequencies were calculated by subtracting resting frequencies from those measured with the current injection. Sodium currents were recorded in voltage clamp mode. All reagents were obtained from Sigma.

RNA Sequencing (RNA-Seq) and gene expression analysis

Total RNA was extracted from five different samples of cells at different stages using the RNAeasy

Micro Kit (Qiagen). The samples included P5 NESCs, P24 NESCs and P36 NESCs, P36 NESC-DN (P36 NESCs had been spontaneously differentiated for 13 days) and induced RGPCs (NESCs cultured in NESC media without CHIR99021 for 7 days). mRNA samples were purified using Oligotex Direct mRNA Kits, according to the manufacturer's instructions (Qiagen). 500 ng mRNA of each sample was used to produce its respective sequence library. mRNA was fragmented using the fragmentation Buffer (Ambion) and double-stranded cDNA was then synthesized using SuperScript II (Invitrogen) RT and random primers. Sequencing was then performed in a HiSeq 2500 apparatus (Illumina) according to the TruSeq RNA Sample Preparation Guide (Illumina). RNA sequencing was performed with the paired end 2x100nt multiplex program. The whole sequencing process was controlled by the Data Collection Software. At least 20M raw reads were taken for every sample. All reads were then mapped using the reference of ensemble macaca_mulatta MMUL_1.72. Mapping ratios of all samples were higher than 90%. Over 16,971 genes were detected with expression in at least one sample. Gene expression analysis was performed by the following methods. Scaled expression levels were used to visualize the expression profiles via heatmap. All transcripts were clustered into 10 modules using k-means. Module names were converted to color names by an R package "WGCNA" (Langfelder and Horvath, 2008). Module functions were analyzed via Database for Annotation, Visualization and Integrated Discovery (DAVID). Based on WGCNA, module eigengenes (MEs) that represent the first principle component of one module were calculated, and related MEs to cell types were used to investigate cell-type related gene modules. To reveal hub genes of a module, the intra-module connectivity were calculated, and the top 30 highly connected genes in each module were defined as hub genes. The coexpression network of hubgenes and functional related genes was visualized via ViSANT software.

For FA dependence experiments, NESCs were digested into single cells and $5.0x10^4$ cells/well were

replated on laminin-coated 6-well plates. The NESCs media were changed into RPIM 1640 NESCs media without FA or with 4.5 μ M FA on the next day, respectively. After 8 days, NESCs were collected to purify RNA, respectively. Two replicates were independently performed. RNA sequencing was then performed based on the above-mentioned protocol. Transcript abundances were quantified using Cufflinks 2.2.1. ~15000 genes were detected with expression in at least one sample. Among which, ~13000 genes were detected with average FPKM>0.1. To evaluate the overall similarity in gene expression, a data matrix of log-transformed the above FPKM values were used for sample-wise hierarchical clustering. The hierarchical clustering analysis was performed using average linkage method and Pearson's correlation as the distance metric. Differential Expression Analysis was performed with linear models as implemented in Limma R package to call significant differentially expressed genes between folic acid treated and untreated cells. ~6000 genes with adjust P value <0.05 were used for the following K-means analysis using Cluster 3.0 to reveal the different clusters or modules of genes with similar gene expression patterns. Euclidean distance was used as the similarity metric. Java tree View was used for the cluster graphing. The Database for Annotation, Visualization and Integrated Discovery (DAVID) was then used to identify enriched Gene Ontology terms within each cluster or module identified by K-means analysis.

Live imaging of NESCs interkinetic nuclear migration in a single cell-derived NT

Replication-defective retrovirus encoding a GFP was produced by co-transfecting a retroviral vector (pMx-GFP from Addgene) with the packaging plasmid pCL (Imgenex, San Diego) in 293T cells. Single NESCs-derived colonies at Day 7 were infected with a retrovirus containing pMx-GFP vector for 4 hours. Then, the media was replaced with fresh NESCs culture media. Live imaging was performed using a Leica TCS SP8 confocal laser scanning system (Leica) equipped with 37^{o}C temperature and 5% CO_2 control. Day 14 single NESCs-derived colonies with NT structures containing GFP-positive cells were imaged with a

40× objective. Time-lapse images were captured at 4 min intervals for 5–10 h.

Cell transplantation

To obtain single NESC-derived cell lines labeled by GFP, NESCs were infected by lentivirus containing GFP and performed clonal expansion. Single-derived GFP positive colonies were selected to continually expand. Four GFP positive cell lines were established and one cell line was randomly chosen for following transplantation.

All surgical procedures and manipulation processes were conducted according to guidelines of the Use and Care of Experimental Animals of the International Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and the Kunming Biomed International Animal Care Committee. Two new born health cynomolgus monkeys (about one month, male), with a body weight ranged from 500 to 800g, were used in the present study. Monkeys were firstly anesthetized with katamine (10 mg/kg) and pentobarbital (10 mg/kg), following preanesthetic medication of atropine (0.1mg/kg) and morphine (0.5mg/kg). They were then placed into a monkey stereotaxic instrument for surgery. Both monkeys underwent a single surgery with a intracranial unilateral injection of single cell-derived NSECs cells labeled by GFP. The unilateral injection was performed in left visual cortex (10μL, $2x10^5$ cells per μL). After surgery, monkeys were given antibiotics for 1 week. Each monkey was housed with his mother in one cage, respectively. The light/dark cycle for these new-born monkeys is 10h/14h.

Tissue processing

Animals were euthanatized with Katamine (10 mg/kg) and pentobarbital (100 mg/kg) before cardiac perfusion with 0.01M PBS. Fresh cold 4% PFA was used to perfuse the monkey (Sigma, PH7.4). Brains were then extracted, post-fixed in 4% PFA (2-3 days) and dehydrated in sucrose solution (10%, 20% and 30%, respectively). The transplanted sites were eventually sectioned on a cryostat microtome at 20μm after

embedding in O.C.T (Sakura-Finetek) and stained with GFP and the neuron or astrocyte antibodies (See Supplemental Table 1). Based on the original sites of injection, cell migration and axon outgrowth in brain sections was measured with a microscale. Every four sections, one section was used to examine GFP cells and stained with neuronal antibodies. The proportion of GFP-labeled cells stained with lineage-specific phenotype markers and a nuclear label (DAPI) was determined by Leica SP8 confocal microscopy. Two thousands or more cells were scored for each marker, such as NeuN, GFAP, vGLUT1 and GABA.

Statistical analysis

All of experiments including immunohohistochemistry were at least performed triplicates. For quantifying neurons, TUJ1 positive cells with a neuronal morphology (a circular, three-dimensional appearance that extend a thin process at least three times longer than their cell body) were quantified as neurons after 2 weeks of differentiation. Quantification was performed on randomly selected 5-8 pictures taken under Leica microscopy using the Leica software package and at least one thousand cells were counted for every experiment. All quantifications are based on at least three independent experiments. Quantification data represented as mean ± standard deviation (s.t.d) using Microsoft Excel STDEV Function. The significance difference between two samples was evaluated by the unpaired two-sample Student's *t*-test using Excel software. $P<0.05$ was considered as statistical significant differences.

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