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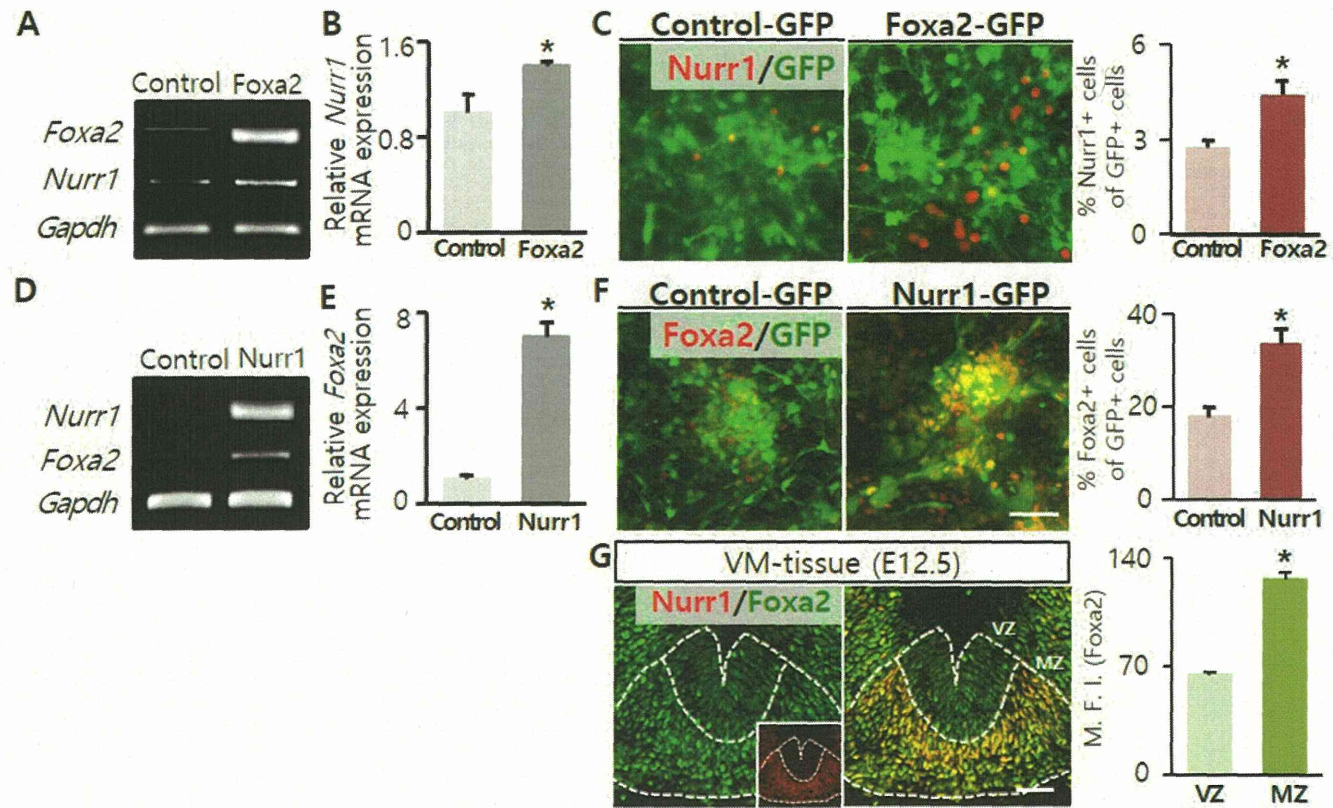
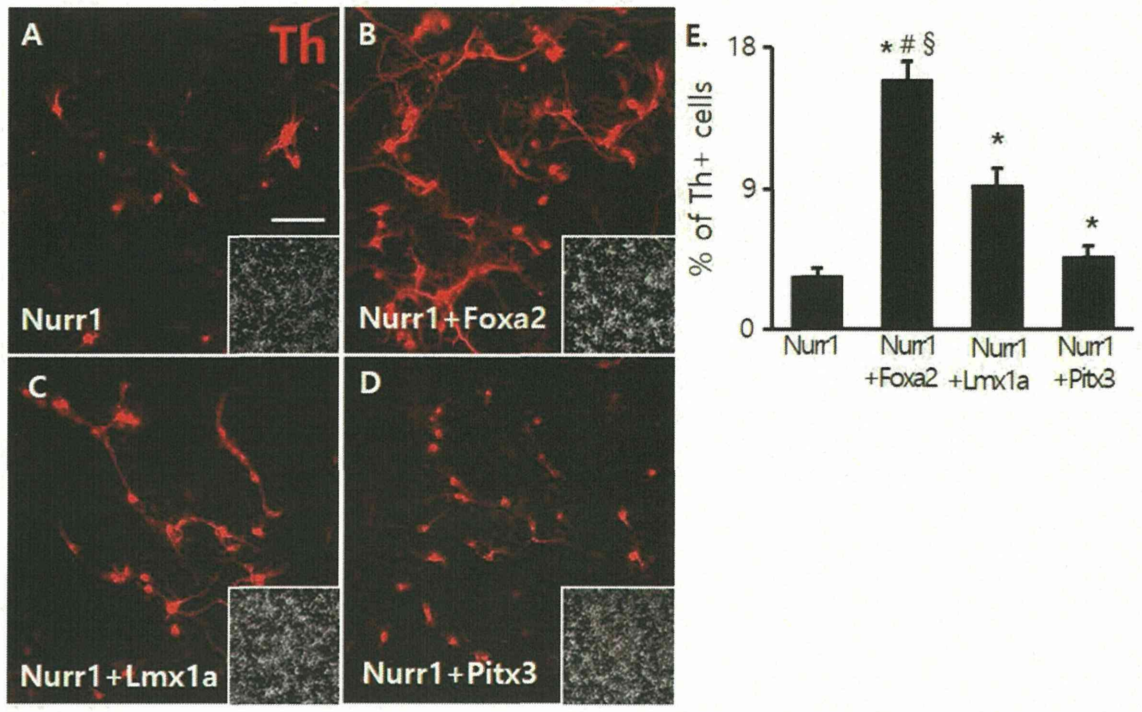


Suppl. Fig. S1. Gain-of-function analyses *in vitro* to confirm positive cross-regulatory loop between Nurr1 and Foxa2 expression. NPCs derived from VM at E11 were subcultured to examine gain-of-function effects. (A-F) The passaged VM-NPC cultures yielded fewer Nurr1⁺ and Foxa2⁺ cells upon differentiation than unpassaged cultures. The passaged cultures were transduced with virus expressing Foxa2-IRES-GFP (A-C) or Nurr1-IRES-GFP (D-F) and differentiated for 6 days. Control cultures were transduced with virus expressing IRES-GFP. Semi-quantitative PCR (A,D), real-time PCR (B,E), and immunocytochemistry (C,F) analyses were carried out for Nurr1 and Foxa2 expression. (C,F) are representative images for Nurr1⁺/GFP⁺ and Foxa2⁺/GFP⁺ cells, respectively. Graphs on the right depict percentages of the co-expressing cells out of total GFP⁺ cells in 20-40 clusters randomly selected from 3 independent culture sets. *Significantly different from control at $P<0.05$(B), $P<0.005$(C), and $P<0.001$(E,F). (G) Comparison of Foxa2 expression levels in the Nurr1-negative ventricular zone (VZ) and Nurr1-positive mantle zone (MZ) of the embryonic mouse VM. VM tissue sections of mouse embryos at E12 were stained with anti-Foxa2 antibody (Inset, identical section Nurr1-stained). Foxa2-stained cells were randomly selected from the VZ and MZ (40 cells each) and Foxa2 expression levels were quantified as mean fluorescence intensities (MFI) of individual anti-Foxa2-stained cells. *$P<0.001$, Student's *t*-test. Scale bar, 50 μm.

Suppl. Fig. S2. Midbrain-type DA neuronal phenotypes of TH$^+$ cells induced by exogenous Nurr1 and Foxa2 co-expression. Non-dopaminergic cortical NPCs were transduced with Nurr1$^+$ Foxa2, and differentiated for 6 days. Immunofluorescence staining was conducted using the indicated antibodies. Scale bar, 50μm.

	Nurr1/Pitx3	Nurr1/HuC/D	Nurr1/NeuN
Pearson's Correlation	0.705±0.045	0.122±0.037	0.325±0.132
Overlap Coefficient	0.799±0.073	0.46±0.038	0.565±0.038

Suppl. Fig. S3. Analysis of Nurr1 colocalization with Pitx3, NeuN, and HuC/D by Pearson's correlation and overlap coefficient values. Shown are representative images of single nucleus co-stained with Nurr1/Pitx3, Nurr1/NeuN, and Nurr1/HuC/D from the VM tissue sections at E12. Scale bar, 5μm.



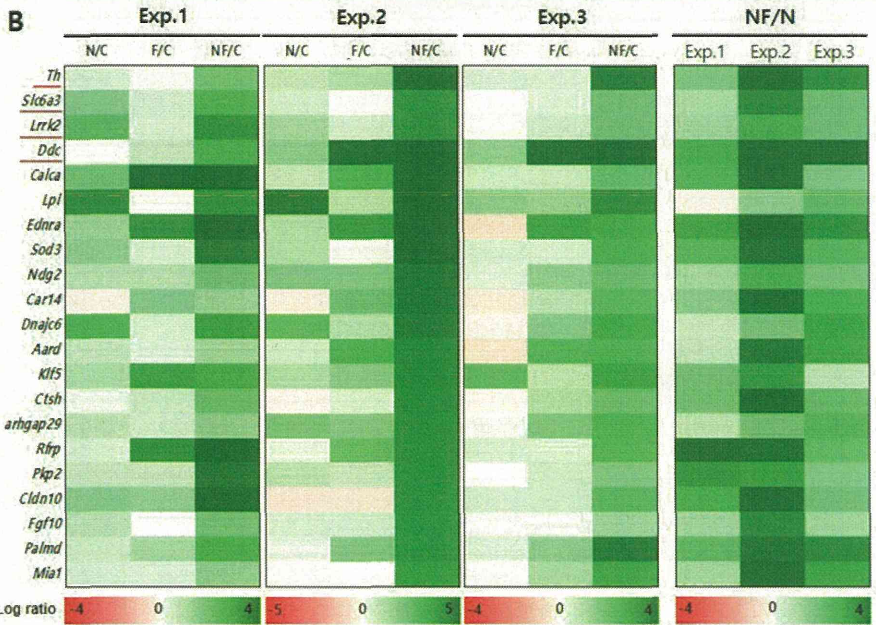
Suppl. Fig. S4. Comparison of the activities of Nurr1 coactivators reported. NPCs derived from mouse embryonic cortices at E12 were co-transduced with the retroviruses containing the control empty vector (A), Foxa2 (B), Lmx1a (C), or Pitx3 (D) along with Nurr1. The coactivator virus titers were carefully adjusted to $1x10^{11}$ virus particles/ml. Graph E represents the percentage of $DAPI^{+}$ cells that were TH^{+}. Significance from the control*, Nurr1+Lmx1a# and Nurr1+Pixt3§ at *P*<0.001, Scale bar, 50μm.

Suppl. Table. S1. mRNA expressions of 21 genes selected from microarray data

A

SYMBOL	DEFINITION	Accession No.	Fold Increase			
			N/C	F/C	NF/C	NF/N
Th	*Tyrosine hydroxylase*	NM_009377	1.71±0.02	1.52±0.43	38.47±1.18	22.513±0.41
Slc6a3	*Dopamine transporter, Dat*	NM_010020	1.23±0.14	-0.03±1.12	12.2±2.81	9.77±1.2
Lrrk2	*Leucine-rich repeat kinase 2*	NM_025730	1.96±0.6	1.22±0.02	12.01±3.63	6.13±0.01
Ddc	*Dopa Decarboxylase, Aromatic L-amino acid decarboxylase, Aadc*	NM_016672	1.66±0.59	26.96±4.83	39.15±6.34	14.3±1.41
Sod3	*Superoxide dismutase3*	NM_011435	1.96±0.22	0.04±1.12	23.36±7.23	11.67±2.37
Calca	*Calcitonin/calcitonin-related polypeptide, alpha*	NM_007587	1.86±0.29	4.71±3.3	28.61±14.5	17.04±10.49
Lpl	*Lipoprotein lipase*	NM_008509	27.16±1.77	6.41±4.7	53.7±6.31	2.0±0.36
Ednra	*Endothelin receptor type A*	NM_010332	1.59±0.45	9.2±3.7	24.5±9.72	14.88±1.85
Ndg2	*Nur77 downstream gene 2*	NM_175329	3.11±0.98	2.58±0.96	16.42±8.79	4.88±1.28
Car14	*Carbonic anhydrase 14*	NM_011797	-1.9±0.53	3.09±1.69	17.18±6.5	24.35±8.42
Dnajc6	*DnaJ (Hsp40) homolog, subfamily C, member 6*	NM_198412	4.23±2.53	2.12±0.35	18.85±2.55	6.31±3.18
Aard	*Alanine and arginine rich domain containing protein*	NM_175503	1.3±0.06	5.86±2.21	14.85±3.25	11.34±1.91
Klf5	*Kruppel-like factor 5*	NM_009769	6.66±4.48	2.2±0.83	17.67±0.17	4.8±3.26
Ctsh	*Cathepsin H*	NM_007801	0.16±1.34	1.3±0.24	12.67±4.31	12.76±7.19
Arhgap29	*Rho GTPase activating protein 29*	NM_172525	3.26±1.39	2.96±0.52	13.73±1.93	4.85±1.49
Rfrp	*Neuropeptide VF precursor*	NM_021892	1.39±0.36	3.15±2.05	14.23±0.09	11.0±2.95
Pkp2	*Plakophilin 2*	NM_026163	1.8±0.17	1.35±0.1	10.72±4.06	5.8±1.71
Cldn10	*Claudin10*	NM_021386	-1.65±0.09	-1.34±0.35	12.22±2.49	16.92±3.86
Palmd	*Palmdelphin*	NM_023245	1.31±0.24	3.26±0.43	26.49±12.01	19.19±5.65
Fgf10	*Fibroblast growth factor 10*	NM_008002	1.33±0.04	1.12±0.06	10.13±3.74	7.7±3.04
Mia1	*Melanoma inhibitory activity 1*	NM_019394	0.06±1.26	0.35±1.56	15.14±2.85	14.26±0.44

B



High through-put gene expression analyses were done on NPCs transduced with control (C), Nurr1 (N), Foxa2 (F), and Nurr1+Foxa2 (NF). To know co-activator role of Foxa2 in Nurr1-induced gene expression, the microarray data were analyzed for the gene expressions up-regulated (>2 folds) in NPCs expressing Nurr1+Foxa2, compared to those expressing Nurr1 alone. 21 genes fit this criterion and are listed with their expression ratios (A) and heatmaps of log2 transformed expression ratios (B). n= 3 independent microarray analyses. Each microarray analysis was done in the control-, Nurr1-, Foxa2-, Nurr1+Foxa2-transduced cultures, and interested gene expressions in the Nurr1-, Foxa2, and Nurr1+Foxa2-expressing cultures were compared with those of the control culture. Genes associated with DA neuron phenotypes are underlined.

Suppl. Table. S2. PCR primers information used in this study

Gene symbol	Sequence	Product size	Cycles & Annealing temp.
PCR primers for gene expression			
Tyrosine Hydroxylase (Th)	F : gccgtctcagagcaggatac R : agcatttccatccctctcct	196bp	30-32 cycles 60 ℃
Dopamine transporter (Dat)	F : tggcttcgttgtcttctcct R : cagctggaactcatcgacaa	221bp	26-28 cycles 58 ℃
Vesicle monoamine transporter2 (Vmat2)	F : ctttggagttggttttgc R : gcagttgtggtccatgag	300bp	26-28 cycles 58 ℃
Engrailed1 (En1)	F : tcaagactgactacagcaacccc R : ctttgtcctgaaccgtggtggtag	200bp	26-28 cycles 58 ℃
Gbx2	F : atgagcgcagcgttcccgccg R : cggcggtggcggcagcacca	200bp	26-28 cycles 58 ℃
CoREST	F : cacttggtatggacgacacg R : cagcccttaggcagaatgag	210bp	30-32 cycles 60 ℃
Forkhead box protein2 (Foxa2)	F : gacataccgacgcagctaca R : ggcaccttgagaaagcagtc	215bp	26-28 cycles 58 ℃
Nurr1	F : cggtttcagaagtgcctagc R : ttgcctggaacctggaatag	194bp	26-28 cycles 58 ℃
Gapdh	F : ctcatgaccacagtccatgc R : ttcagctctgggatgacctt	154bp	25-28 cycles 60 ℃
PCR primers for ChIP assay			
1. Primers for *Foxa2* promoter			
Foxa2 (Region1)	F : ctgcaggcagagaacacaga R : ctttctggctacccacctca	248bp	40-45 cycles 58 ℃
Foxa2 (Region2)	F : caagaccctccactccaaaa R : cagaggcaggaggatctcag	193bp	40-45 cycles 58 ℃
2. Primers for *Nurr1* promoter			
Nurr1 (Region1)	F : gcggtgggtcattgtttc R : gcgctccggttcattgtc	199bp	40-45 cycles 58 ℃
Nurr1 (Region2)	F : gggcacagtggcttaaaagt R : ctcctctgcaagttccaacc	181bp	40-45 cycles 58 ℃
Nurr1 (Region3)	F : tgaataagacacgcgtcagg R : agccccactgtcctttcttt	212bp	40-45 cycles 58 ℃
Nurr1 (Region4)	F : cagtgtcttaggggccagag R : gaagatcagctactctgctgga	221bp	40-45 cycles 58 ℃

Suppl. Table. S3. Prediction of Nurr1 and Foxa2 binding sites on promoters of DA neuronal marker genes.

TF	Gene promoter (Kb from TSS)	PWM setting		Predicted binding sites			
				Mouse		Rat	
				Sequence	Location	Sequence	Location
Nurr1	*Foxa2* (M:-1085) (R: -1069)	Conservation cutoffs	94%	AAGCTCAC	420~427	AAGCTCAA	428~435
				GTAACCTT	1041~1048	GTAACCTT	1025~1032
		Window size	50				
		Score threshold	80%				
	Th (M:-2505) (R: -2461)	Conservation Cutoffs	70%	AAGGTTAA	356-363	AAGGTTAA	336~343
				GAGGACAC	1399~1406	GAGGACAC	1346~1353
		Window size	50	AAGGTCCC	1511~1518	AAGGTCCC	1454~1461
		Score threshold	80%	GAGGTCAG	1788~1795	GAGGTCAG	1747~1754
				CTGGCCTT	2437~2444	CTGGCCTT	2392~2399
	Dat (M:-2775) (R: -2556)	Conservation Cutoffs	70%	CTGACCTA	560~567	TTGACCTA	246~253
				GTGACCAT	2045~2052	GTGACCAT	1820~1827
		Window size	50	GAGACCTG	2184~2191	GTGACCTG	1955~1962
		Score thresold	75%	GTGGCCTC	2643~2650	GTGGCCTC	2423~2430
Foxa2	*Nurr1* (M:-1091) (R: -1010)	Conservation Cutoffs	46%	AATGCAAATGA	212~223	ATACCAAAGAGC	129~140
				GACTGATAATTG	238~249	GAATGTGCAGGG	151~162
		Window size	50	AAATATTTACCT	370~381	CCCCGTTTCCCT	200~211
		Score threshold	60%	AAGCCCCTTTAG	389~400	AAGCATCCTGTG	219~230
	Th (M:-2505) (R: -2461)	Conservation Cutoffs	80%	ACACAGACAAAG	230~241	ACACAGACAAAG	211~222
				AAAGCAATATTT	320~331	AAAGCAATATTT	300~311
				CAATATTTGTGT	324~335	CAATATTTGTGT	304~315
		Window size	50	AAATCCACATTC	362~373	AAATCCACACTC	342~353
				GAGCAGGCAGTG	826~837	GAGCAGGCAGTG	783~794
				GAGTAAATAGTC	840~851	GAGTAAATAGTC	797~808
		Score threshold	85%	GAGTAGATAGTA	2000~2011	GAATAGATAGTA	1964~1975
				CTAGATTTATTT	2094~2105	CTAGATTTGTCT	2063~2074
				AATCCAGCATGG	2151~2162	AATCCAGCATGG	2109~2120
	Dat (M:-2775) (R: -2556)	Conservation Cutoffs	80%	GAATAAATGTTT	1357~1368	GAATAAATGTTT	1017~1028
				AAATGTTTGTTG	1361~1372	AAATGTTTGCTG	1021~1032
		Window size	50				
		Score threshold	85%				

by HATs causes the remodeling of chromatin from a tightly to a loosely packed configuration, leading to transcriptional activation. Conversely, a decrease of histone acetylation by HDACs results in a condensed chromatin structure and thus suppresses transcription (Juliandi et al., 2010). Inhibition of HDAC activity by valproic acid (VPA), a widely used anticonvulsant and mood-stabilizing drug, has been shown to drive mESCs to differentiate into the ectodermal lineage at the expense of mesodermal and endodermal lineages (Murabe et al., 2007). This ectodermal lineage differentiation is further biased in favor of neuronal rather than glial fates by the VPA treatment (Murabe et al., 2007). Prior to this finding in mESCs, we and others (Hsieh et al., 2004; Balasubramaniyan et al., 2006) had also found a similar tendency for neuronal over glial fate preference when we cultured neural progenitor cells (NPCs) in the presence of HDAC inhibitors such as VPA and trichostatin A. The types of neurons produced in these studies were not examined in detail, however, and the effects of HDAC inhibition on the differentiation of mESC-derived NPCs have not yet been studied.

Here we report that HDAC inhibition in mESC-derived NPCs treated with VPA resulted in a recapitulation of later stages of corticogenesis. VPA treatment increased the production of cut-like homeobox 1 (Cux1)-positive superficial-layer neurons and decreased that of B-cell leukemia/lymphoma 11B (Bcl11b; also called Ctip2)-positive deep-layer neurons. These results suggest an important role of histone acetylation for the specification of superficial-layer neurons in late corticogenesis.

2. Materials and methods

2.1. Maintenance of mESCs

The mESC line 46C (*Sox1*-GFP-IRES-pac knock-in) was routinely propagated without feeder cells as described previously (Ying et al., 2003; Conti et al., 2005). mESCs were grown at 37 °C in a 5% (v/v) CO_2 incubator in ESC medium (ESM) containing Glasgow Minimum Essential Medium (Invitrogen), supplemented with 10% (v/v) fetal bovine serum (Biowest), 1 mM sodium pyruvate (Invitrogen), 0.1 mM MEM non-essential amino acids (Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma), and 1000 U/ml murine leukemia inhibitory factor (Millipore), on 0.1% (v/v) gelatin-coated (Sigma) 9-cm dishes (Nunc). Medium was changed every day, and when the cells reached 60–70% confluence they were passaged onto new dishes at a plating density of 1×10^6 cells per 9-cm dish.

2.2. Neural differentiation

mESCs were induced to differentiate to the neural lineage as described previously (Ying et al., 2003; Conti et al., 2005; Gaspard et al., 2008, 2009). In brief, mESCs were trypsinized, dissociated and plated on 0.1% (v/v) gelatin-coated (Sigma) dishes at a density of 0.3×10^6 cells per 9-cm dish (Nunc) in ESM. One day later, the medium was replaced with DDM, which is composed of DMEM/F12 (Invitrogen) supplemented with freshly prepared modified N2-supplement (Ying and Smith, 2003), 1 mM sodium pyruvate (Invitrogen), 0.1 mM MEM non-essential amino acids (Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma), 0.5 mg/ml bovine serum albumin fraction V (Invitrogen), 1× GlutaMAX (Invitrogen) and 0.5× antibiotic–antimycotic (Invitrogen). This day was designated as differentiation day 0. Cyclopamine (Calbiochem) was added to a final concentration of 1 μM from differentiation day 2 to day 10. On differentiation day 10, the medium was replaced with DDM only (without cyclopamine). For selection of Sox1-expressing neural progenitor cells, from differentiation day 8 to day 10, puromycin (Sigma) was added to a final concentration of 0.5 μg/ml. The culture was maintained until differentiation day 12 and the medium was changed every 2 days during day 0 to day 12.

At differentiation day 12, mESC-derived NPCs were trypsinized, dissociated and plated on poly-L-lysine/laminin-coated (Sigma, Becton Dickinson) dishes at a density of 0.5×10^6 cells per 3.5-cm dish (Nunc) in N2/B27 medium, which consists of a 1:1 mixture of DDM (without sodium pyruvate and MEM non-essential amino acids) and Neurobasal/B27 medium (Neurobasal, 1× B27 supplement without vitamin A, 1× GlutaMAX and 0.5× antibiotic–antimycotic (all from Invitrogen)). Valproic acid (Sigma) was added one time to the culture medium to a final concentration of 0.5 mM at differentiation day 12 where appropriate. Culture was maintained until differentiation day 14 or 21, and the medium was changed every 2 days during day 12 to day 21.

2.3. Immunocytochemistry

Medium was removed and cells were washed with phosphate buffered saline (PBS) and then fixed with 4% paraformaldehyde in PBS for 15 min. After 3 washes with PBS, the cells were incubated for 1 h at room temperature (RT) in blocking solution (PBS containing 3% FBS and 0.1% Triton X-100). They were then incubated overnight at 4 °C with the appropriate primary antibodies. The following primary antibodies were used: chick anti-GFP (1:500, Aves Labs), rabbit anti-β-tubulin isotype III (Tuj1; 1:1000, Covance), mouse anti-nestin (1:250, Millipore), rabbit anti-Pax6 (1:500, Covance), mouse anti-Map2ab (1:1000, Sigma), rat anti-Ctip2 (1:1000, Abcam), mouse anti-reelin (1:1000, MBL), and rabbit anti-Cux1 (1:500, Santa Cruz). After 3 washes with PBS, the cells were incubated for 2 h at RT with the appropriate secondary antibodies. The following secondary antibodies were used: FITC-conjugated donkey anti-chick, Cy5-conjugated donkey anti-rabbit, Cy3-conjugated donkey anti-rabbit, Cy5-conjugated donkey anti-mouse, Cy3-conjugated donkey anti-mouse (all 1:500, Jackson ImmunoResearch), Alexa Fluor 488-conjugated donkey anti-mouse, Alexa Fluor 488-conjugated donkey anti-rabbit, and Alexa Fluor 488-conjugated donkey anti-rat (all 1:500, Invitrogen). After 3 washes with PBS, nuclei were stained for 15 min at RT with Hoechst 33258 (Nacalai Tesque). Cells were washed with PBS, mounted on cover slips with Immu-Mount (Thermo Scientific), and examined and photographed using a fluorescence microscope (Axiovert 200M, Zeiss) equipped with a camera and appropriate epifluorescence filters.

3. Results

3.1. 46C mESCs differentiate into the neuroectodermal lineage under Shh inhibition

We used 46C mESCs, one of whose characteristic features is the replacement of the *Sox1* open reading frame with that encoding green fluorescent protein (GFP) (Ying et al., 2003). Since *Sox1* is the earliest known neuroectodermal marker in the mouse embryo (Pevny et al., 1998; Wood and Episkopou, 1999), we could follow neural commitment of 46C mESCs in culture by monitoring their GFP expression.

46C mESCs can differentiate efficiently into the neural lineage in feeder-free adherent monolayer culture supplemented with serum-free medium (Ying et al., 2003; Conti et al., 2005; Abranches et al., 2009). *Sox1*-GFP-expressing NPCs can be detected from differentiation day 2, and composed more than 75% of the total cell population at differentiation day 4 in N2/B27 medium (Ying et al., 2003). To assess the neural commitment of 46C mESCs in the adherent monolayer culture system proposed by Gaspard et al. (2008), we cultured these cells at low density in a chemically defined

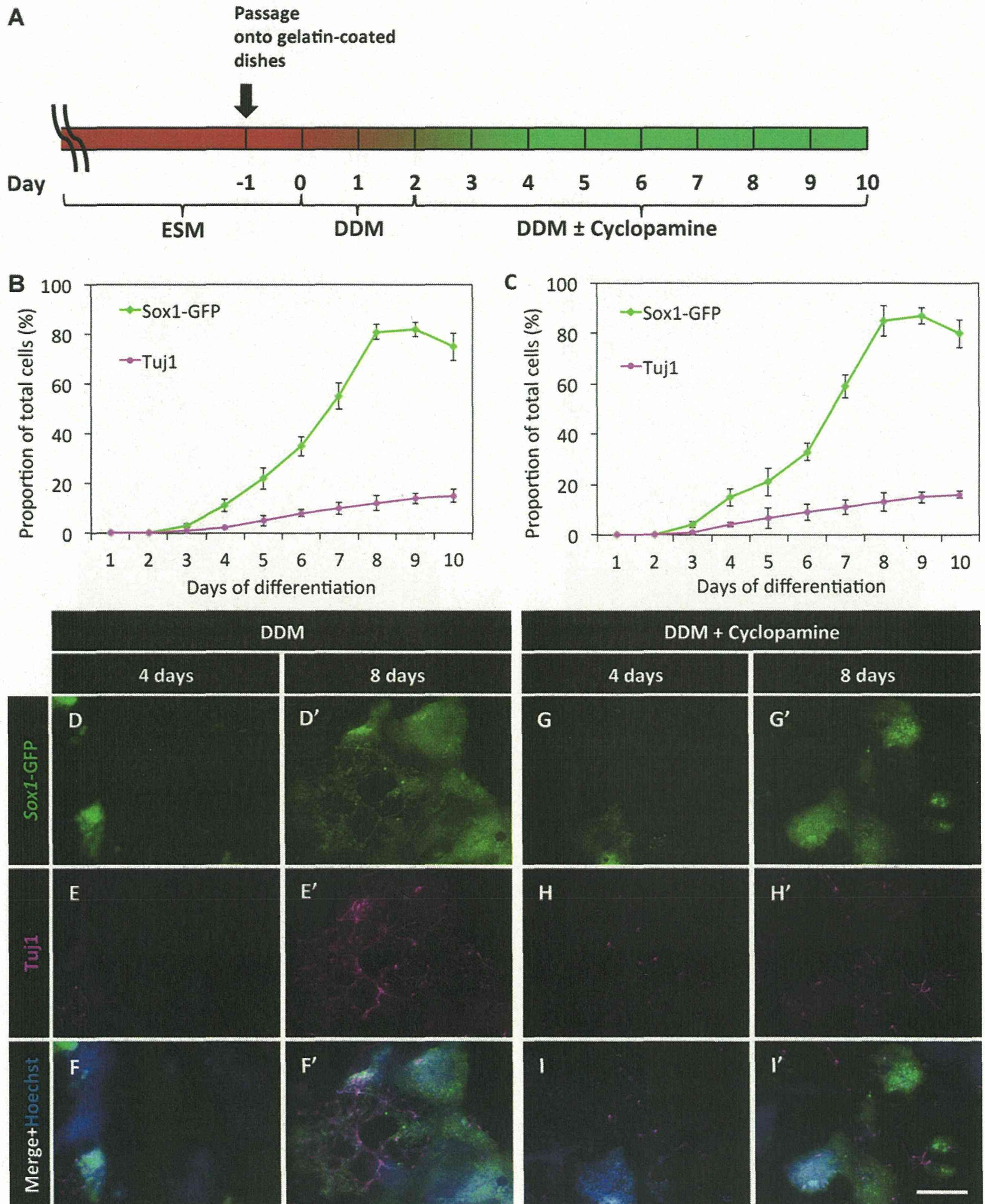
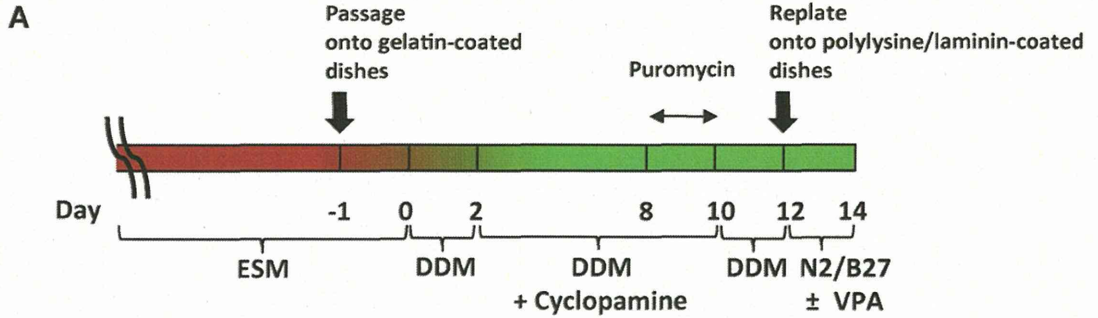


Fig. 1. 46C mESCs differentiate to the neuroectodermal lineage. (A) Timeline of the neural induction protocol using chemically defined default medium (DDM). mESCs were routinely propagated and then passaged to gelatin-coated dishes in embryonic stem cell medium (ESM) 1 day before neural induction (day −1). The next day (day 0), the medium was changed to DDM, and cyclopamine was added where appropriate from differentiation day 2 to day 10. The proportions of *Sox1*-GFP+ NPCs and Tuj1+ neurons did not differ in DDM (B) and in DDM with cyclopamine (C) during the culture period. Data are mean ± SD from at least three independent experiments. (D–I) Representative immunostaining images from differentiation day 4 and day 8 of *Sox1*-GFP-expressing (green in D, D′, G, and G′) and Tuj1-expressing (magenta in E, E′, H, and H′) cells, used for the quantitative data shown in B and C. Merged images with Hoechst (blue in F, F′, I, and I′) are also shown. Scale bar is 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)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Passage
onto gelatin-coated
dishes
Puromycin
Replate
onto polylysine/laminin-coated
dishes
Day
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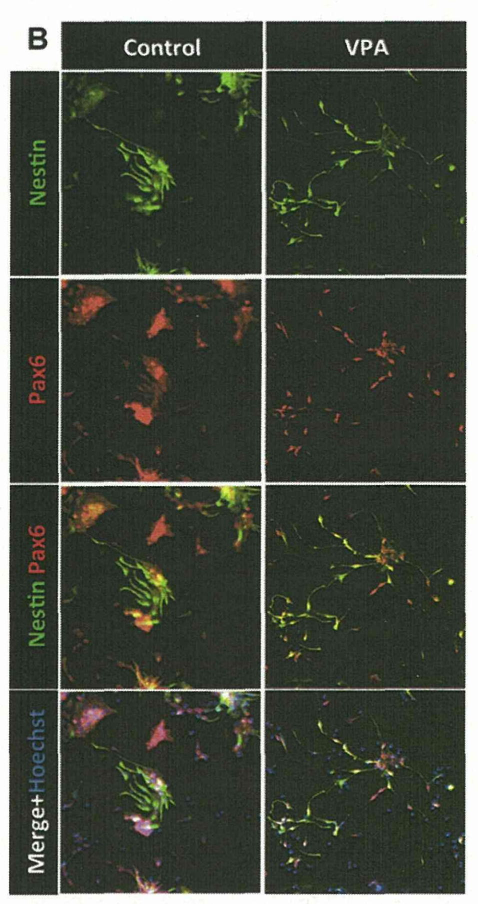
ESM
DDM
DDM
+ Cyclopamine
DDM
N2/B27
± VPA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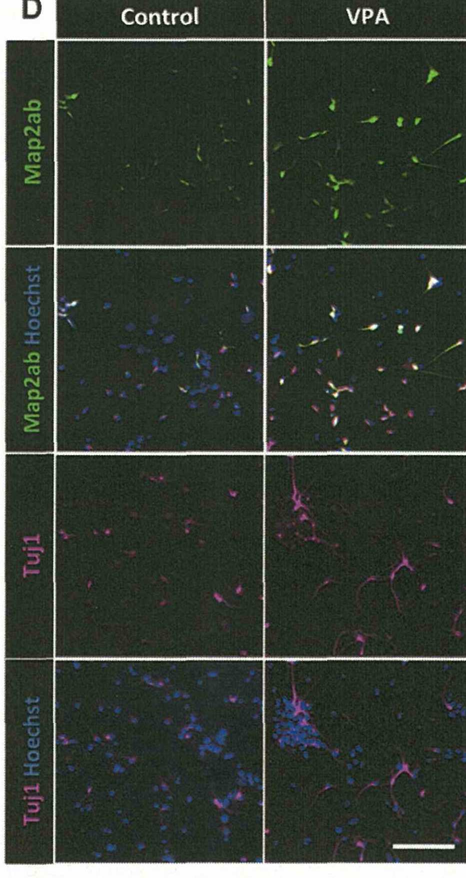
VPA
Nestin
Pax6
Nestin Pax6
Merge+Hoechst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VPA
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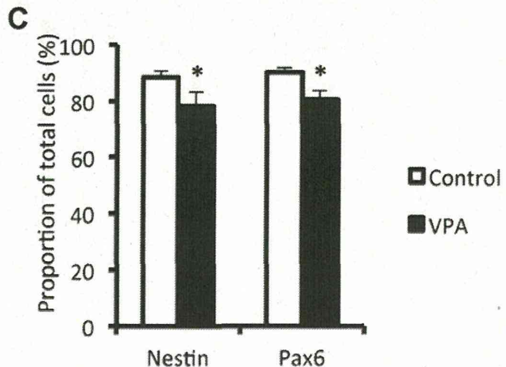
Tuj1
Tuj1 Hoechst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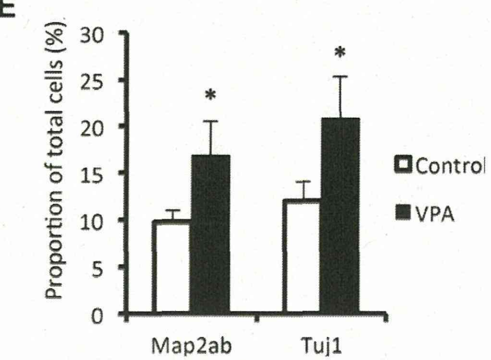
Proportion of total cells (%)
100
80
60
40
20
0
Nestin
Pax6
Control
VPA
*


E
Proportion of total cells (%)
30
25
20
15
10
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